FAILURE TO INACTIVATE UREASE WITH NEAR-INFRARED RADIATION.

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In 1932 Collier and Wasteneys reported that crude preparations of urease were inactivated by near-infrared radiation. They used glycerol extracts of jack bean meal, to obtain "conditions approximating the natural conditions as nearly as possible," and treated them with infrared for four hours at 30° C. Since that time, infrared has been reported to have a variety of effects upon systems of biological interest. It is known to sensitize chromosomes to breakage (Swanson and Yost, 1951) or mutation (Swanson, Hollaender and Kaufmann, 1948) by X-rays or ultraviolet radiation, cause abnormal cleavages in *Urechis* (Nelson and Brooks, 1935), destroy nicotinic acid in the presence of hydrogen peroxide (Brown, Thomas, and Bina, 1946), etc. These findings lend new interest to the effect of infrared in the enzyme systems of cells. For this reason, studies were undertaken to determine the effect of combined radiations on urease.[2]

METHODS.

Three preparations of urease were used in this study: a 0·1 p.c. solution of Arlco urease; a crude glycerol extract diluted 1 in 74 with doubly distilled water or with buffer (pH 7·0); and recrystallized (3X) urease prepared by the method suggested by Sumner (1951) from jack bean meal kindly supplied by Prof. Sumner. The purified preparation was diluted before use to give an activity comparable to that of the other preparations. Infrared radiation was delivered from a General Electric heat lamp, through a Corning No. 2540 filter and one cm. of water in a constant temperature bath. The transmission of this system ranged from 7500 to 15,000 Å. The temperatures of the enzyme solution and of the water bath (15° C. or 30° C.) were always within one degree. The enzyme was contained in a 50 ml. Pyrex test tube, rotated slowly, 15 cm. from the infrared source. Ultraviolet radiation was carried out at room temperature, using rotating quartz flasks kept 25 cm. from a 15-watt General Electric germicidal lamp. The ultraviolet exposure was 30 minutes for all tests and caused about 50 p.c. inactivation of the preparations. Gamma radiation was delivered from a 440 curie Co^{60} source filtered with aluminum. The dose throughout was 1000 r, as measured by a Victoreen dosimeter with gamma ray chamber. The gamma

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ray treatment resulted in about 30 p.c. inactivation of the preparation. Hydrogen peroxide treatments were made by addition of 0·4 p.c. Merck Superoxol to the diluted enzyme solution 15 minutes prior to assay. In all cases, the controls were given the same treatment as the test material with the exception of the exposure to the radiation itself. Activity was determined colorimetrically by the method of Sizer (1939). The studies were carried out at several different hydrogen ion concentrations (pH 7·0 and others). As there was no difference in the response to infrared radiation, the data are lumped.

RESULTS.

The data in Table 1 show quite clearly that none of the urease preparations was inactivated by near-infrared radiation. These data are in direct contradiction to those of Collier and Wasteneys (1932), who report an inactivation of

TABLE 1.

Exposure of different preparations of urease to various doses of near-infrared radiation. Infrared delivered at temperatures indicated. Data expressed as mg NH_3 N/ml/30 min.; presented as averages of several determinations with standard deviations.

Treatment	30°C.			15°C.	
	Arlco	glycerol extract	recryst.	glycerol extract	recryst.
Control	·41 ± ·01	1·00 ± ·13			
IR 3 hrs.	·45 ± ·08	·90 ± ·01			
Control	·60 ± ·04	1·06 ± ·01		·94 ± ·01	·52 ± ·04
IR 4 hrs.	·69 ± ·06	1·06 ± ·01		·92 ± ·03	·53 ± ·02
Control	·62 ± ·02	·97 ± ·08	·43 ± ·02	1·03 ± ·05	·46 ± ·04
IR 18 hrs.	·67 ± ·04	1·02 ± ·07	·44 ± ·03	1·01 ± ·12	·48 ± ·02

TABLE 2.

Exposure of urease preparations to various combined treatments. Infrared delivered 4 hours at temperatures indicated. Data expressed as NH_3 N/ml/30 min.; presented as averages of several determinations with standard deviations.

Treatment	30°C.		15°C.	
	glycerol extract	recryst.	glycerol extract	recryst.
UV 30 min.	·59 ± ·12	·25 ± ·06	·52 ± ·03	·22 ± ·03
IR + UV	·53 ± ·09	·26 ± ·02	·54 ± ·04	·26 ± ·04
0·4% H_2O_2	·36 ± ·05	·22 ± ·02	·38 ± ·01	·23 ± ·02
IR + H_2O_2	·37 ± ·02	·21 ± ·03	·35 ± ·03	·23 ± ·02
1000r γ		·24 ± ·02		·36 ± ·07
IR + γ		·24 ± ·02		·40 ± ·11

23 p.c. with 4 hours treatment at 30° C. Lower temperatures also were tried in this study as it has been shown that the infrared effect upon chromosomes is low-temperature dependent (Swanson and Yost, 1951). However, there is no significant effect at either temperature. Since it was possible that the effect

might be dependent upon the presence of another radiation or oxidant (Swanson and Yost, 1951; Brown *et al.*, 1946), several combined treatments were tried. Table 2 presents these data. It can be seen that in no case is there a significant change in the inactivation when infrared is used as a pretreatment. Each figure in the Tables represents an average of at least three separate experiments. In the case of Table 1, 18 hours of infrared, each figure represents at least seven different trials.

DISCUSSION.

It is evident from the data presented in this paper that no effect of near-infrared radiation on urease has been found. The discrepancy between these data and those of Collier and Wasteneys (1932) cannot be accounted for on the basis of dosage. The intensity reported by those authors is $\cdot 96 \times 10^{-3}$ cal./cm^2/sec. Although the intensity of our source was not measured for these studies, it is known from previous work (Yost, 1952) that the intensity is in excess of 13×10^{-3} cal./cm^2/sec. Since an 18-hour exposure in our apparatus was unable to cause deactivation, it is unlikely that the difference is one of dosage.

It is possible that the effect observed by Collier and Wasteneys (1932) was due to an impurity in the preparation, which was absent in ours, and which absorbed the infrared and transmitted the energy to the urease molecule. It is also possible that what was measured was the thermolability of the enzyme, although they report temperature control. More likely is the possibility that the control and test preparations of Collier and Wasteneys had different amounts of protein or other stabilizing materials, thus resulting in lower activity of the test preparation. In any case, the data presented here show that there is no effect upon the urease molecule itself (as shown by the crystalline preparation) or on the crude material prepared by the authors.

SUMMARY.

Several preparations of urease were treated with near-infrared radiation alone and in combination with other radiations or oxidants. In no case was there inactivation of the preparations which could be related to the infrared. These data are in direct contradiction to a previous report of the inactivation of urease by infrared radiation.

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THE DETERMINATION OF VITAMIN B_{12}-ACTIVITY IN THE ORGANS AND EXCRETA OF SHEEP

4. THE SEPARATION OF VITAMIN B_{12}-ACTIVE FACTORS FROM RUMEN CONTENTS BY PAPER IONOPHORESIS

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A study of the vitamin B_{12}-activity of the rumen contents of sheep has already been reported (Hine and Dawbarn, 1954). In all the samples of rumen contents examined the greatest measure of activity was given by a plate assay using a mutant of *Escherichia coli* as the test organism, while progressively lower values were obtained by three tube assays with *E. coli* mutant, *Lactobacillus leichmannii* 313 and *Ochromonas malhamensis*.

The nature of the diet was reflected in the level of activity in the rumen contents but the four measurements were differently affected by it. Sheep grazing on normal pasture had a much larger proportion of the total vitamin B_{12}-activity of the rumen contents in a form which could be utilized by *Ochromonas* than did sheep receiving cobalt deficient wheaten-hay chaff and gluten with added cobalt. When the cobalt supplement was withdrawn from the latter diet the overall activity in the rumen contents declined sharply and the ratio between the measurements made by the four methods also changed.

The vitamin B_{12}-activity in the rumen contents and faeces of sheep and cattle, in the faeces of pigs and in sewage sludge has been shown to be due to the presence of cyanocobalamin (vitamin B_{12}) and other related substances, factor B, pseudo-vitamin B_{12}, factors A, C, G, H, factors E and F, factor III and others. The chemical constitution of some of these substances has been determined and found to differ from vitamin B_{12} itself only in the nature of the nucleotide component of the molecule. Factor B is denucleotided vitamin B_{12}. The base in the nucleotide of cyanocobalamin is 5:6-dimethylbenziminazole, in pseudo-vitamin B_{12} it is adenine, in factor A it is 2-methyladenine and in factor III it is 5-hydroxybenziminazole (Pfiffner, Dion and Calkins, 1952; Brown and Lester Smith, 1954; Gant, Lester Smith and Parker, 1954; Pfiffner, Calkins and Dion, 1954; Brown, Cain, Gant, Parker and Lester Smith, 1955; Ford, Holdsworth

and Kon, 1955; Johnson, Bentley and Moxon, 1956; Neujahr, 1956; Shunk, Robinson and Folkers, 1956).

The present communication describes the use of paper ionophoresis to separate the vitamin B_{12}-like factors in the rumen contents of sheep grazing on normal pasture or pen-fed on wheaten-hay chaff and given cobalt by mouth.

METHODS.

Samples of rumen contents were mixed with about six times their weight of glass distilled water containing cyanide, the reaction was adjusted to pH 5 and the mixture was steamed for 30 minutes. After cooling, the insoluble residue was removed by centrifuging (Hine and Dawbarn, 1954).

Purification of extracts.

Freshly prepared extracts were usually opalescent but on alternate freezing and thawing precipitation occurred leaving a relatively clear supernatant solution. Extracts clarified in this way were used for paper ionophoresis. This method of purification was extremely simple and could be carried out without loss of activity. Purification of the active factors in the extracts by adsorption on charcoal and elution by refluxing with 65 p.c. acetone was always accompanied by some loss of activity and a change in the ratio of activity as measured by the different assay procedures. The resulting material was therefore no longer representative of the rumen contents from which it was derived. Moreover, extracts treated in this way showed no cleaner separation of the peaks of activity by paper ionophoresis than did those clarified by freezing and thawing. In consequence the only method of purification used in the following work was alternate freezing and thawing.

Preparation of the paper for ionophoresis.

The paper used was washed in a dilute solution of sodium acetate containing cyanide. A sheet of Whatman No. 1 filter paper measuring 61 cm. square was suspended in a chromatography cabinet with the upper edge of the paper dipping into a glass trough filled with a solution containing per litre 1·36 gm. Na acetate $3H_2O$, 12·5 mg. KCN and sufficient acetic acid to bring the reaction to pH 6·8-6·9. After 48 hours' washing with this solution the paper was washed with glass distilled water for a further 48 hours, and was then dried in air at room temperature and cut into pieces 11 cm. wide.

The solutions to be examined by ionophoresis were applied to the paper by means of a micrometer syringe, while a stream of air at room temperature hastened drying. Three spots were put on each paper, 3·5 cm. apart, along a line 20 cm. from one end.

Separation of vitamin B_{12}-active factors by paper ionophoresis.

The method used was based on that of Holdsworth (1953). The apparatus was the same as that described by Foster (1952). The solvent was 0·5 N acetic acid containing 0·01 p.c. KCN. The paper was placed in the apparatus with the short (20 cm.) end towards the anode. After ionophoresis with a potential difference of approximately 1125 volts applied for 6 hrs. the paper was dried in air at room temperature in the dark. It was then cut into three longitudinal strips, 3 cm. wide, one for each spot. A margin, 0·5 cm. wide, outside and between the strips was discarded. The strips were then cut into pieces 1 cm. long, each of which was placed in an assay tube and the vitamin B_{12}-activity was measured either by the *E. coli* tube or the *L. leichmannii* assay. The paper pieces were soaked overnight in a buffer of the same composition as that used to wash the paper (v.s.). The volume used was 2·5 ml. for the *E. coli* assay and 5 ml. for the *L. leichmannii* assay.

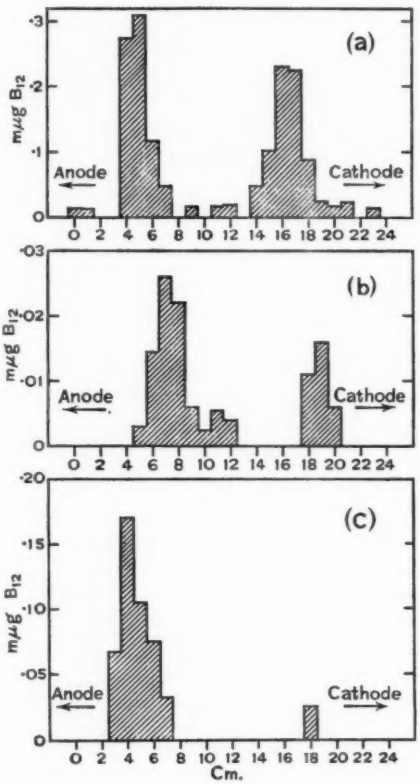

Fig. 1. The separation of vitamin B_{12}-active factors from the rumen contents of sheep by ionophoresis on paper.

Sheep No. 1110, pen-fed on wheaten-hay chaff and gluten.

1 mg. Co *per os* per day.

Vitamin B_{12}-activity in rumen contents extract

	mμg./ml.
E. coli plate assay	82
E. coli tube assay	15·7
L. leichmannii assay	12·3
Ochromonas assay	7·9

(a) *E. coli* tube assay.

Ochromonas activity as p.c. of *E. coli* tube activity in the extract, 50 p.c.

B_{12}-activity located at -4 to -7 cm. as p.c. of total B_{12}-activity on the paper, 47 p.c.

(b) *L. leichmannii* assay.

Ochromonas activity as p.c. of *L. leichmannii* activity in the extract, 64 p.c.

B_{12}-activity located at -5 to -10 cm. as p.c. of total B_{12}-activity on the paper, 64 p.c.

(c) *Ochromonas* assay.

B_{12}-activity located at -3 to -7 as p.c. of total B_{12}-activity on the paper, 95 p.c.

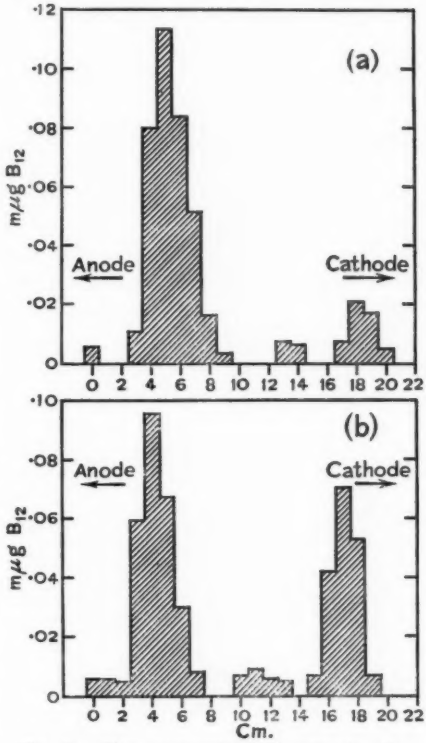

Fig. 2. The separation of vitamin B_{12}-active factors from the rumen contents of sheep by ionophoresis on paper.

L. leichmannii assay.

(a) Sheep on normal pasture.

Vitamin B_{12}-activity in rumen contents extract

	mμg./ml.
E. coli plate assay	123
E. coli tube assay	51
L. leichmannii assay	47
Ochromonas assay	38

Ochromonas activity as p.c. of *L. leichmannii* activity, in the extract, 81 p.c.

B_{12}-activity located at -3 to -9 cm. as p.c. of total B_{12}-activity on the paper, 84 p.c.

(b) Sheep No. 446, pen-fed on wheaten-hay chaff and gluten.

1 mg. Co *per os* per day.

Vitamin B_{12}-activity in rumen contents extract

	mμg./ml.
E. coli plate assay	260
E. coli tube assay	35
L. leichmannii assay	23
Ochromonas assay	13·8

Ochromonas activity as p.c. of *L. leichmannii* activity, in the extract, 60 p.c.

B_{12}-activity located at 0 to -7 cm. as p.c. of total B_{12}-activity on the paper, 57 p.c.

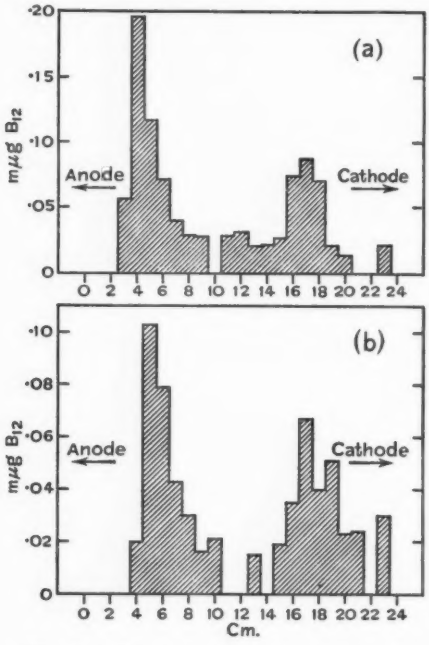

Fig. 3. The separation of vitamin B_{12}-active factors from the rumen contents of sheep by ionophoresis on paper.

Sheep No. 1110, pen-fed on wheaten-hay chaff and gluten.

1 mg. Co *per os* per day.

E. coli tube assay.

(a) Rumen contents extract 0·05 ml.

(b) Rumen contents extract 0·025 ml. + factor B solution 0·025 ml. Factor B alone appeared at − 21 to − 23 cm.

It was found that the buffer caused a small increase in the response of the micro-organisms to vitamin B_{12}. Accordingly the solutions of pure vitamin B_{12} used to obtain the standard dose response lines were made up in the same buffer solution. In the *E. coli* assay the paper caused a slight lowering of the dose response line. Pieces of paper, 1 cm. × 3 cm., were therefore added to all standard tubes in this assay.

After the paper pieces had been soaked overnight assay medium was added to each tube without removing the paper. From this point the assays were carried out by the usual procedure and the vitamin B_{12}-activity of the contents of each tube was determined.

A few tests have been carried out using the *Ochromonas* assay to measure the vitamin B_{12}-activity. This organism is sensitive to acetate (Ford, 1953a). We have found that even 0·003 N acetate is slightly toxic and 0·04 N acetate inhibits growth completely. The paper pieces were therefore not soaked in acetate buffer for these tests. Since they would necessarily contain some acetate from the ionophoresis solution, paper similarly treated was added to all the standard tubes. Water and assay medium were added to the tubes containing paper on the morning of assay.

Preparation of factor B.

A solution of factor B, denucleotided cyanocobalamin, was prepared by mixing 10 ml. conc. HCl and 1 ml. vitamin B_{12} solution, each at 65° C., and maintaining the mixture at 65° C. for 5 minutes. After cooling most of the HCl was distilled off under reduced pressure, a trace of cyanide was added and the pH was adjusted to about 6·8. This solution no longer showed any activity towards *L. leichmannii* but supported the growth of *E. coli* in the plate and tube assays. We concluded that the whole of the cyanocobalamin had been destroyed and that part of it had been converted to factor B. No doubt other degradation products were present in the solution, but these were not expected to show any microbiological activity (Gant *et al.*, 1954; Lester Smith, 1955).

This solution was subjected to paper ionophoresis alone and mixed with an extract of rumen contents. The resulting paper strips were tested for vitamin B_{12}-activity by the *E. coli* tube method of assay.

RESULTS AND DISCUSSION.

The results of these experiments have been presented diagrammatically in Figs. 1-3. In each case the ordinate gives a measure of the vitamin B_{12}-activity derived from a single piece of paper, while the abscissa indicates the excursion from the origin under the influence of the electric current or due to electro-endosmosis.

Sheep on a diet of wheaten-hay chaff and gluten given cobalt by mouth.

The vitamin B_{12}-activity of the extracts of rumen contents was separated by ionophoresis on paper into two major components, with a subsidiary peak of activity between them, and, for *E. coli* only, a small additional peak nearer the cathode. This last is considered to be due to factor B. When a solution containing factor B was added to the rumen contents extract before examination by ionophoresis this peak was magnified (Fig. 3). The solution of factor B by itself appeared on the paper in this same position.

The first large concentration of activity is due either to cyanocobalamin or to some factor which is not separated from it by ionophoresis, and which is, moreover, active for *Ochromonas* (Fig. 1, c). When a solution of pure vitamin B_{12} was mixed with the extract of rumen contents the magnitude of this peak was increased. By either the *E. coli* tube or *L. leichmannii* assay, the proportion of the total activity found in the first major component was approximately the same as the proportion of the activity in the extract which was available to *Ochromonas* (Figs. 1 a and b, 2 a and b).

It seems likely that the second main concentration of activity is attributable to factor A. It occupies a position on the paper which might be expected for factor A (Ford, Holdsworth, Kon and Porter, 1953; Holdsworth, 1953). Factor A is more active for *E. coli* than for *L. leichmannii* and shows a trace of activity for *Ochromonas* (Ford, 1953 a and b; Brown *et al.*, 1955). This is also true of the activity on the paper at this point (Fig. 1, a, b, c). Ford, Holdsworth and Kon (1955) have reported that factor A is the most abundant vitamin B_{12}-active factor in the rumen contents of calves.

Sheep grazing on normal pasture.

The distribution of active factors in the rumen contents of sheep on pasture presents a marked contrast to that of the previous group of animals (Fig. 2, a). This is due to a great decline in the activity of the second major component and is in keeping with the observation repeatedly made that in the rumen contents of sheep grazing on normal pasture a large proportion of the total vitamin B_{12}-active material can be utilized by *Ochromonas*.

It is not surprising that the proportions of the different vitamin B_{12}-active factors in the rumen should be dependent on the diet of the sheep. Studies *in vitro* have shown that microorganisms which are capable of synthesizing vitamin B_{12} analogues can be directed towards the synthesis of a particular

analogue by supplying the appropriate intermediate in the culture medium. *E. coli* 113-3 in a medium containing factor B produced factor C. When the medium contained 5:6-dimethylbenziminazole as well as factor B it produced cyanocobalamin, while factor B and riboflavin led to the synthesis of factor C and cyanocobalamin (Ford *et al.*, 1955). Factor B was not necessary for the synthesis of vitamin B_{12} analogues by *Streptomyces griseus*. This organism, like *E. coli*, could be influenced in its production of the analogues, by the intermediates supplied in the medium (Fantes and O'Callaghan, 1955 and 1956).

SUMMARY.

The vitamin B_{12}-active factors in the rumen contents of sheep have been separated by ionophoresis on paper. The distribution of the factors throughout the paper was determined by cutting it into pieces and measuring the vitamin B_{12}-activity in each piece by microbiological assays with *Escherichia coli* mutant, *Lactobacillus leichmannii* or *Ochromonas malhamensis* as test organism.

Rumen contents of sheep grazing on normal pasture were contrasted with those of sheep pen-fed on wheaten-hay chaff and gluten with supplementary cobalt.

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THE METABOLISM OF LOWER FATTY ACIDS IN DIABETIC SHEEP

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The greater part of the energy requirements of sheep are met by the lower fatty acids, acetic, propionic and butyric, which are formed by bacterial fermentation in the paunch of ruminants (Marston, 1939, 1948; Phillipson, 1947, 1948). Of these lower fatty acids it is known that acetate is an important source of carbon atoms in numerous synthetic reactions (Block, 1947), propionate is glucogenic (Ringer, 1912; Jarrett, Potter and Filsell, 1952), and butyrate is ketogenic and possibly associated with glucogenesis (Potter, 1952; Kleiber *et al.*, 1954).

The metabolism of acetate is closely linked with the metabolism of carbohydrate, and its subsequent oxidation or condensation is in part determined by the supply of carbohydrate precursors. Jarrett, Potter and Filsell (1952) have indicated that in adult sheep the metabolism of acetate may well depend upon the concurrent metabolism of the glucogenic fatty acid, propionate.

An impairment of carbohydrate metabolism, characteristic of diabetes in non-ruminants, leads to an impairment of the utilization of acetate due to lack of the necessary precursors to form oxaloacetate (Chaikoff, 1952; Ciaranfi and Fonnesu, 1954).

In a diabetic ruminant, however, an adequate supply of propionate should provide the necessary carbohydrate precursors to enable oxidation of acetate to proceed if propionate can readily be converted to the necessary precursors in the absence of insulin.

Severe diabetes has now been shown to occur in sheep after an injection of alloxan (Jarrett, 1946) or after complete extirpation of the pancreas (Jarrett, Potter and Packham, 1956).

In the present study, tolerance curves following an intravenous injection of acetate, propionate or butyrate have been established in normal sheep and compared with those in diabetic sheep. At the same time, the concentrations of sugar, ketone bodies and pyruvate in the blood have been determined in an attempt to elucidate some aspects of the metabolic channels involved in the metabolism of lower fatty acids in normal and diabetic sheep.

METHODS.

Five Merino ewes were made diabetic by injecting a freshly prepared 5 p.c. solution of alloxan monohydrate (50 mg. per kg.). Three other ewes which were made diabetic by pancreatectomy were severely hyperglycaemic with an apparent ketosis in two of them. The eight diabetic animals, together with nine normal sheep, were fed a daily ration consisting of 1,000 gm. of a mixture of lucerne chaff and wheaten chaff in a 2 : 1 ratio. This was offered immediately after the last sampling period each day and was usually consumed by the next morning; any residue was removed. Daily blood and urine analyses were performed to confirm the persistence of the diabetes. An intravenous injection of 5 mM. per kg. of acetic, propionic or butyric acids was given to each diabetic sheep and to the nine normal animals. These solutions were neutralized to pH 7·0 with caustic soda; the volume administered was 100 ml.

A fine plastic tube (1 mm. I.D. and 1·7 mm. E.D.) was inserted for several inches into the jugular vein on the day preceding the experimental period and filled with heparin solution (500 units in saline) to reduce the possibility of blood clots occurring in the tube. Blood samples were obtained with a minimum of handling by attaching the syringe to the end of the tube. This allowed the animal to remain placid throughout the manipulations, a condition which is essential for such experiments as these, in which activity very materially influences the concentration of pyruvic acid in the blood.

Pre-injection blood samples were obtained and the solution then administered over a period of 5-10 minutes. Blood samples were taken subsequently to the injection at 30 and 60 minutes and thereafter at hourly intervals for 5 to 6 hours.

Volatile fatty acids were estimated by a micro modification of the method of Friedmann (1938) using an electrometric titration on the distillate. A modification of the method of Behr and Benedict (1926) was used for the determination of ketone bodies; pyruvic acid was estimated by the method of Friedmann and Haugen (1943) and sugar by the method of Nelson (1944) and Somogyi (1952).

RESULTS.

The five animals which received alloxan were all severely diabetic with blood sugar values between 125 and 270 mg. p.c. and blood ketone values between 11 and 44 mg. p.c. The three depancreatized animals were hyperglycaemic, with blood sugar values between 150 and 200 mg. p.c. Two of these were severely ketotic with blood ketone values of 25 mg. p.c. One of them succumbed four weeks after the operation, and the other was treated with insulin six weeks after the operation, when in a state of collapse. This animal recovered after a daily injection of insulin (80-120 units) which eliminated the ketosis, although complete control of the hyperglycaemia throughout the twenty-four hours was not achieved. The other animal survived for seven months: during the first five months blood ketone values of about 1·5 mg. p.c. were observed and the animal appeared healthy. Ketosis then developed with values of 9 to 11 mg. p.c. of total ketone bodies in the blood and the animal developed the typical signs of severe diabetes and succumbed within eight weeks.

Insulin was not administered at any stage during the period when observations were being made on the diabetic animals.

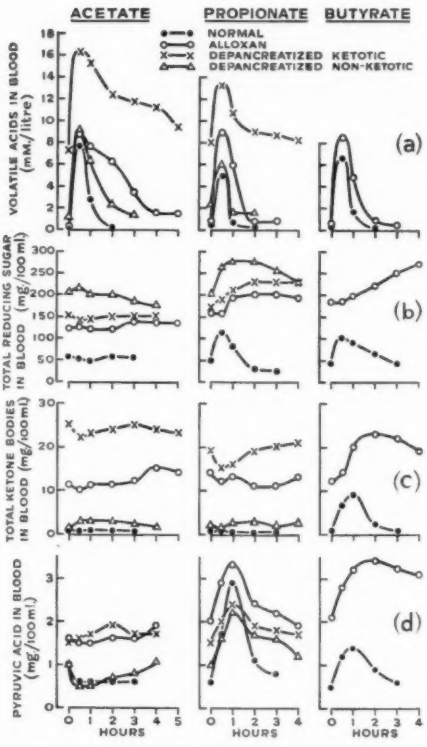

Fig. 1. Blood levels of (a) volatile acid, (b) sugar, (c) ketone bodies, (d) pyruvate, following an intravenous injection of acetate, propionate or butyrate.

When the volatile fatty acids were injected, propionate was removed more rapidly than either acetate or butyrate from the blood of both normal and diabetic sheep. With normal sheep, the level of volatile acid in the blood had returned to the pre-injection level within two hours of the acetate or butyrate injection and within one hour of the propionate injection. In the diabetic animals, however, the level was still raised four to five hours after the acetate injection and two to three hours after the propionate or butyrate. During the period when the depancreatized animal (No. 77) was non-ketotic, the initial concentration of volatile acids in the blood was the same as that of the normal sheep; the tolerance curve for propionate was normal, and for acetate slightly delayed. When the animal became ketotic the initial level was much higher than normal and the tolerance curves were similar to those obtained with the other severely diabetic animals.

Typical tolerance curves for a normal and a diabetic animal following the injection of each of the lower fatty acids are shown in Fig. 1 (a), and the concentration of volatile fatty acids in the blood at the various sampling times are given in Table 1.

The formula $ae^{-bt} + c$ was fitted to the tolerance curves. As ae^{-bt} gives a straight line when plotted on logarithmic × arithmetic graph paper, a value of c, found by trial and error so as to get the points as nearly into line as possible, was substracted from each set of observations, and a line drawn through them; "b" was determined from this line. The half-life characteristic, i.e. the time taken to come half-way back to normal, is $(\log_e 2)/b$. The means of the values of b for normal and diabetic sheep were compared by "t" tests.

This analysis indicated that there was a highly significant difference between the normal and diabetic sheep after both the acetate ($P < \cdot 001$) and

TABLE 1.

Volatile Fatty Acids in Blood (mM/litre) at times of sampling.

	Animal	Acetate							Propionate						Butyrate				
		0	30	60	120	180	240	300	0	30	60	120	180	240	0	30	60	120	180
Normal	41	0·73	6·3	2·9	0·73				0·73	5·6	1·6	0·60	0·66		0·59	4·6	0·44	0·22	
	23	0·83	5·8	2·1	0·75				0·69	4·5	0·50	0·41			0·88	11·0	4·4	0·61	
	38	0·58	7·4	3·2	0·44	0·50			0·41	6·4	0·67	0·29	0·34		0·53	9·0	3·0	0·60	
	61	0·79	5·5	2·4	0·39	0·24			1·3	5·4	0·53	0·67	0·67		0·60	7·3	2·6	1·1	
	79	0·72	5·9	2·0	0·33	0·62			0·90	2·9	0·30	0·42	0·43		0·74	8·6	2·2	0·8	
	88	0·33	5·9	2·2	0·57	0·33			0·88	7·4	1·1	0·81			0·79	11·4	6·8	0·8	
	10	0·55	5·4	2·4	0·44				0·59	7·4	1·3	0·44			0·22	6·1	1·5	0·22	
	62	0·44	9·1	4·4	0·54	0·44			0·44	5·9	0·91	0·59			0·65	10·3	5·6	0·87	
	51	0·33	7·6	2·7	0·22				0·44	4·9	0·65	0·22			0·22	6·6	1·6	0·22	
Alloxan	15	4·2	16·7	15·2	12·0	11·0	9·0	7·7	1·8	10·7	4·7	1·9	1·8	0·7					
	30	1·8	14·1	11·4	8·7	7·1	5·4	3·5	0·94	10·7	5·7	1·2	0·87	0·80	1·3	9·1	5·2	1·5	1·6
	62	1·1	14·7	13·2	10·0	7·5	5·8	3·6	0·83	10·2	5·9	1·3	1·2		0·66	11·3	7·7	1·7	1·3
	81	0·65	13·1	10·5	6·7	3·6	1·7	0·60	0·54	10·8	6·9	2·4	1·1		0·72	12·3	9·3	4·6	1·7
	05	0·22	8·7	7·6	6·2	3·4	1·5	1·5	0·76	8·9	5·9	0·67	0·67		0·43	8·5	4·8	0·87	0·43
	05 (with insulin)	0·48	4·1	0·50	0·39	0·43	0·35		0·10	1·8	0·17	0·15			0·22	5·1	0·43	0·22	
Depan-creatized	44	7·2	16·2	15·2	12·3	11·7	11·2	9·3	8·0	13·3	10·7	9·0	8·7	8·2					
	77	8·6	17·7	15·9	12·4	12·3			7·9	13·9	9·9	8·5	7·3						
	77 (non-ketotic)	1·1	9·1	6·3	2·3	1·4			2·3	6·0	1·6	1·6							

TABLE 2.

Total Reducing Sugar in Blood (mg./100 ml.) at times of sampling.

	Animal	Acetate							Propionate							Butyrate						
		0	30	60	120	180	240	300	0	30	60	120	180	240	300	0	30	60	120	180	240	300
Normal	62	46	54	45	56	56	53	58	45	147	85	50	33	32	44	42	122	73	29	30	40	52
	51	58	51	49	58	56			48	122	81	30	24			43	101	93	66	44		
	41	46	51	49	49	49			42	94	78	67	53			42	101	56	23	28	37	42
	38	55	60	58	56	62			69	134	118	87	71			49	136	109	71	60	53	
	79	52	45	42	45	49			60	144	112	72	44			36	97	55	21	21	30	
Alloxan	15	271	215	193	200	178	193	178	153	181	215	232	215	181	165							
	30	178	193	170	193	205	197	220	170	197	210	237	220	205	220	185	165	189	165	157	143	193
	62	224	189	196	185	193	200	224	220	255	271	255	271	237	237	215	170	160	200	255	266	262
	05	123	125	123	123	137	135	135	157	157	193	200	200	193	197	185	185	197	220	250	271	276
Depan-creatized	44	153	140	147	150	150	151		170	189	210	228	228	228								
	77	189	193	178	193	170			215	237	262	267	255									
	77 (non-ketotic)	205	215	200	200	185	174		200	266	275	275	255	228								

propionate injections ($P = .001$) with a slightly less significant difference ($P < \cdot 05$) after the butyrate injection.

The concentration of the blood sugar did not significantly alter after the injection of acetate in either the diabetic or normal sheep. After propionate injection into the normal animal, a marked rise in the level of blood sugar was apparent within thirty minutes but it had returned to normal in two hours. In the diabetic, however, the rise to a maximum level took place at a much slower rate and the elevated value was sustained for a much longer period. In the non-ketotic diabetic the maximum value was observed earlier than in the ketotic group but the level was still elevated for three to four hours. The injection of butyrate into the normal sheep consistently produced a rise in blood sugar; whereas in the three alloxan diabetic animals which received butyrate, the results were variable.

Blood sugar curves following the injection of the three fatty acids into typical animals are presented in Fig. 1 (b), and details of each animal presented in Table 2.

In the normal sheep the injection of acetate resulted in an increase in the concentration of ketone bodies in the blood, butyrate produced a much greater increase and propionate brought about a slight reduction in the level of circulating ketones. The results obtained with the diabetic sheep were somewhat equivocal. Butyrate produced a significant rise in the level of blood ketone bodies which was elevated for a much longer period than that obtained with the normal animals. Any small change produced by acetate and propionate could not be considered significant in view of the large amounts of ketone bodies in the blood of the ketotic diabetics. However, the administration of these acids did appear to produce a similar response in the level of circulating ketone bodies to that observed in normal sheep.

The results are presented in Table 3, and graphs of typical animals in Fig. 1 (c).

The concentrations of pyruvic acid in the blood of the diabetic sheep prior to any fatty acid administration were about twice those of the normal animals. No significant change occurred in the pyruvate levels after acetate injection in either the normal or diabetic sheep.

Propionate injections led to a rise in pyruvate in both the normal and diabetic animals with the maximum value observed one hour after the injection, and a return to normal within two to three hours. A comparison made between normal and diabetic groups indicated that there was a tendency for the normal animals to show a greater rise in pyruvate, but statistical analysis indicated that the difference was not quite significant due largely to one animal in the normal group (No. 51) behaving differently from the other four animals.

An injection of butyrate caused a slight rise in blood pyruvate in the normal animals and a significantly greater rise in the diabetics. This rise in the diabetics

TABLE 3.

Total Ketone Bodies in Blood (mg./100 ml.) at times of sampling.

	Animal	Acetate							Propionate						Butyrate					
		0	30	60	120	180	240	300	0	30	60	120	180	240	0	30	60	120	180	240
Normal	62	0·91	2·0	1·5	1·2	0·93			1·1	0·85	0·68	0·45	0·79		1·5	8·8	11·6	6·5	3·2	2·1
	51	0·60	0·82	0·96	0·74	0·63			0·58	0·61	0·30	0·31	0·53		0·60	6·8	9·0	2·4	0·81	
	41	0·74	4·5	5·0	2·7	2·2			1·4	1·2	1·2	0·90	1·6		1·2	8·5	8·5	2·6	2·6	
	79	1·6	3·5	2·3	1·9	1·6			3·5	3·0	1·2	1·4	2·8		2·1	10·4	11·6	3·8	2·4	
Alloxan	30	30	28	27	31	34	36	36	28	28	28	33	35	34	32	36	41	40	42	40
	62	45	42	47	47	47	47	49	44	44	49	44	42	42	47	49	53	55	55	58
	05	11	10	11	11	12	15	14	14	12	13	11	11	13	12	14	20	23	22	19
Depan-creatized	44	25	22	23	24	25	24	23	19	15	16	19	20	21						
	77	11	12	12	15	12			11	6	6	9	9							
	77 (non-ketotic)	1·7	2·8	2·8	2·8	2·3	1·7		2·3	1·7	2·8	2·8	2·3	2·8						

TABLE 4.

Pyruvic Acid in Blood (mg./100 ml.) at times of sampling.

	Animal	Acetate						Propionate						Butyrate					
		0	30	60	120	180	240	0	30	60	120	180	240	0	30	60	120	180	240
Normal	62	0·5	0·4	0·6	0·8	0·7	0·5	0·6	1·7	2·9	1·1	0·8	0·7	0·5	1·2	1·2	1·2	0·9	0·6
	51	1·0	0·6	0·6	0·6	0·6		0·5	1·2	1·1	0·5	0·5		0·5	1·2	1·4	0·9	0·6	
	79	1·2	1·3	1·5	1·0	1·5		0·8	3·6	3·8	1·6	1·5		0·3	0·9	1·1	0·6	0·6	
	38	1·5	1·2	1·4	1·1	1·1		1·3	4·6	5·5	2·2	1·3		0·5	1·5	1·5	0·9	0·6	
	41	1·1	0·8	1·1	1·1	1·4		1·0	2·4	2·7	1·0	0·8		0·6	1·3	1·3	0·9	0·6	
Alloxan	30	1·6	1·8	1·9	2·7	2·6	2·6	1·9	2·3	2·2	2·9	2·7	2·6	1·7	2·6	2·9	3·0	2·6	2·3
	62	2·6	2·7	3·0	3·2	3·3	3·3	2·2	3·1	3·6	3·2	2·7	2·9	3·1	3·9	4·3	5·3	4·7	4·4
	05	1·6	1·5	1·5	1·6	1·6	1·9	2·0	2·9	3·3	2·4	2·2	1·9	2·1	2·8	3·2	3·4	3·2	3·1
Depancreatized	44	1·5	1·6	1·7	1·9	1·7	1·7	1·5	2·0	2·4	1·9	1·8	1·7						
	77 (non-ketotic)	1·0	0·5	0·5	0·7	0·8	1·1	1·0	1·6	2·2	1·7	1·6	1·2						

was sustained for three to four hours. The pertinent data are presented in Table 4, and graphs illustrating the response of typical animals shown in Fig. 1 (d).

After the injections of butyrate the normal sheep became distressed with respiratory discomfort and partial collapse. Butyrate was administered to only three of the diabetic animals because of this distress which led to the death of one animal; the other two were seriously affected.

When insulin was administered to control the diabetes in an alloxan diabetic and a depancreatized animal, the response to injected fatty acids was the same as that observed for normal sheep.

DISCUSSION.

Diabetes can be produced in sheep by either pancreatectomy or by an intravenous injection of alloxan. A diabetogenic dose of alloxan always gives rise to a severe ketosis and hyperglycaemia, whereas pancreatectomy may, in some animals, lead to hyperglycaemia without concurrent ketosis (Jarrett, Potter and Packham, 1956).

In all diabetic sheep a slow rate of removal of injected volatile fatty acids from the systemic circulation was found to be a characteristic feature. The tolerance curves obtained after an acetate, propionate or butyrate injection indicate that the delayed return to the pre-injection level is more pronounced with acetate than with propionate or butyrate, and that sheep with ketosis exhibit this phenomenon to a greater extent than do the depancreatized animals without ketosis.

The results suggest an impaired utilization of the lower fatty acids in diabetic sheep. The sustained high level of volatile fatty acids, which was regularly found in the blood of the depancreatized animals with ketosis, indicates a greater impairment of fatty acid utilization in these animals than in those made diabetic with alloxan.

Although the tolerance curves obtained after propionate and butyrate injections indicate some delay in the utilization of these acids, the observed rise in blood sugar suggests at least a partial incorporation of these acids into the metabolic pool in diabetic sheep. This rise to a maximum value takes place more slowly than in normal sheep and is sustained for a much longer period (4-5 hours). The latter effect could be associated with a delay in the utilization of sugar, a known feature of diabetic ruminants as shown by glucose tolerance curves (McCandless, Woodward and Dye, 1948; Jarrett, Potter and Packham, 1956).

The data obtained from analyses of the blood levels of ketone bodies and pyruvate after injections of propionate did not provide sufficient information to assess the extent to which diabetic sheep can utilize propionate, but it does appear that some failure or delay in propionate metabolism is present in diabetic ruminants.

After butyrate the rise in ketone bodies and pyruvate was significantly greater and of longer duration in the diabetic than in the normal. The sustained elevation of pyruvate could be the consequence of an alteration in carbohydrate metabolism in the diabetic and it is possible that the ketone body response may also be a reflection of this effect.

When diabetic sheep are adequately controlled with insulin the response to injected fatty acids is similar to that of normal animals, and indicates that the delay in fatty acid utilization in the diabetic is due in some way to a lack of insulin.

In normal sheep the metabolic route of acetate probably depends on the availability of other metabolic intermediaries. The rise in ketone bodies which occurs after acetate injection is considerably modified by the simultaneous injection of propionate and it has been suggested that propionate, in providing the necessary glucogenic intermediaries, is important in determining the subsequent fate of acetate (Jarrett, Potter and Filsell, 1952).

It is now generally accepted that oxidation of fatty acids through the tricarboxylic acid cycle depends on the availability of coenzyme A and oxaloacetate, an intermediary which can be readily supplied from the breakdown of carbohydrate. Deficiency of oxaloacetate leads to ketosis. A metabolic block in glycolysis, or failure of glucose to enter the cell—two possible consequences of insulin lack (Stadie, 1954)—would inhibit the production of oxaloacetate and thereby affect the metabolic fate of acetate, leading to ketosis.

Ruminants, however, probably rely on propionate and on gluconeogenesis from protein for the supply of oxaloacetate rather than from carbohydrate. If a block in glycolysis exists in diabetic ruminants then the supply of propionate which is probably metabolized through pyruvate (Mahler and Huenneckens, 1953) or succinate (Flavin, Ortiz and Ochoa, 1955) should provide the necessary 2 C. units to enable the oxidation of acetate to proceed if there is no direct effect of insulin at the tricarboxylic acid cycle level. Hence, if propionate can be used for oxaloacetate synthesis in diabetic ruminants no ketosis should ensue provided there is no other effect of insulin at this stage.

The facts that severe ketosis does occur in some diabetic ruminants, and that an impaired utilization of acetate and propionate is evident, indicate that in the absence of insulin the entry of propionate into the tricarboxylic acid cycle is impaired. Thus, in view of the diabetic syndrome in depancreatized sheep in which hyperglycaemia is always present, but ketosis is not an invariable feature, it seems likely that some other factor apart from a block in glycolysis is involved.

SUMMARY.

Tolerance curves following the intravenous injection of acetate, propionate and butyrate have been established in normal and diabetic sheep.

Sheep made diabetic by an injection of alloxan or by pancreatectomy show a delayed removal of the lower fatty acids from the systemic circulation.

The delay in the removal of injected acetate is more pronounced than that of propionate or butyrate.

Concurrent observations on the blood concentrations of sugar, ketone bodies and pyruvate following the injection of the lower fatty acids indicate partial failure in the utilization of these acids by diabetic ruminants.

It is suggested that some other factor besides a metabolic block in glycolysis is present in depancreatized sheep.

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ECTROMELIA VIRUS MULTIPLICATION IN REGENERATING MOUSE LIVER

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The reproduction of most viruses seems to follow a similar pattern; adsorption of the virus to the host cell, an eclipse period during which part or all of the infectivity disappears and finally reappearance of the infecting virus in increasing titre and its liberation to the exterior. Many authors have suggested that the eclipse period may hold the key to the understanding of the nature of virus multiplication. Studies of the growth cycle of ectromelia virus in mouse liver (Nossal and de Burgh, 1954) have suggested that during the eclipse period a preliminary step occurred which was concerned in establishing control over one or more of the cell's synthetic centres. The work described below deals with some effects of ectromelia virus on host cell activities during the eclipse period of the growth cycle. The pattern of ectromelia virus multiplication in regenerating liver has been studied as well as the effect of such multiplication on the regenerative growth of the liver cells. A preliminary report of this work has been given in a previous paper from this laboratory (de Burgh and Miller, 1955).

Materials and Methods.

Virus.

The 'Moscow' strain of ectromelia virus was used in all the experiments. Stock suspensions were made by grinding infected livers in cold mortars and homogenizing in 10 p.c. sucrose so as to make a 1 in 5 suspension. After a clearing spin at 5,000 revolutions per minute for 10 minutes, the supernatant was kept in stoppered tubes in the deep freeze at $-15°$ C. The titre of such suspensions was over $10^{9.6}$ $L.D._{50}$ per ml. and remained unaltered for over two months.

Stock suspensions made in an identical manner but from normal non-infected livers were labelled "normal homogenate" and used for control purposes.

Mice.

Albino mice weighing between 10 and 30 gm. were used for partial hepatectomy and growth curve experiments. Two series were used for partial hepatectomy; A in which all the mice were males and less than 3 months old; B in which the mice were of different age and sex.

Albino mice or heterogenetic coloured mice of the Walter and Eliza Hall Institute (Melbourne) strain were used for bioassays of infectivity.

Partial hepatectomy.

The operation performed under ether anaesthesia was similar to that described for the rat by Higgins and Anderson (1931). By such a method, it is possible to remove two-thirds of the liver (left and middle lobes). The mice received post-operative injection of 1 mg. streptomycin in 0·2 ml. saline subcutaneously to diminish the risk of post-operative infection. Mice found clinically unwell in the post-operative period were discarded, as were those whose weight, measured 24 hours after operation, was less than 85 p.c. of pre-operative weight.

Estimation of liver restoration.

The mice were killed at various post-operative intervals by decapitation. At both operation and sacrifice, the liver was removed, freed of excess blood, and weighed on a torsion balance to the nearest 5 mg.

In a separate experiment involving 23 albino mice which were partially hepatectomized in the usual manner and then immediately killed to remove the rest of the liver, it was found that the ratio of the liver removed at operation to the total liver was $0{\cdot}658 \pm 0{\cdot}035$ with a coefficient of variation of 0·053. The ratio of the weight of liver to the weight of the body in these mice was $0{\cdot}0488 \pm 0{\cdot}0057$ with a coefficient of variation of 0·12. Hence the former relationship is several times as consistent as the latter, as was found in the rat by Brues, Drury and Brues (1936). An assumption was therefore made that the weight of the liver removed at operation divided by 0·66 represented the weight of the liver before operation. Milligrams of liver excised and mg. of liver found at sacrifice are measured directly. The percentage of regeneration could thus be calculated and was corrected in each case to allow for gain or loss of body weight as a result of operation trauma or normal growth (Brues, Drury and Brues, 1936).

Cytology.

Portions of the liver removed at both operation and sacrifice were fixed in Zenker's fluid containing 5 p.c. glacial acetic acid for routine cytological work and cell counting. They were stained by haematoxylin and eosin or by pyronin-methyl green.

Direct measurement of the number of liver cells in any specimen was made after the method of Brues, Drury and Brues (1936), and the error introduced by the fact that all nuclei visible do not necessarily lie entirely within the section examined but also partly within an adjacent section was corrected (Abercrombie, 1946). The small error resulting from the presence of binucleate cells was disregarded.

Mitoses were counted under the oil immersion objective and over an area containing at least 1,000 hepatic cell nuclei. The incidence of mitoses was expressed as percentage of hepatic cell nuclei dividing in a given area. In some experiments, colchicine was given intraperitoneally in doses of 0·002 mg. per gm. body weight 3 hours before sacrifice in order to arrest mitosis at metaphase.

Growth curves.

Mice at various post-operative intervals were inoculated intravenously with 0·25 ml. of the virus suspension described above and the time of injection accurately recorded. At desired intervals after the injection, the mice were killed by decapitation and the livers treated as above, a portion being weighed, ground up in a chilled mortar, homogenized in 10 p.c. sucrose so as to make a 1 in 10 suspension, stored at − 15° C., and titrated within a week or two.

Virus titration.

Bioassays of infectivity were made using the Reed and Muench (1938) method of calculating the mouse $L.D._{50}$. The standard error was calculated according to the Pizzi (1950) formula and was usually between 0·2 and 0·3 log unit.

Chemical analysis.

The DNA was separated according to the method of Schmidt and Tannhauser (1945), and measured by phosphorus estimation or by absorption at 260 mμ.

RESULTS.

Regeneration of liver in normal mice.

Table 1 shows the amount of regeneration of liver at various times after operation.

The total number of hepatic parenchymal cells had not significantly increased during the first 30 hours after the operation even though the residual liver had doubled its size. Thereafter the number increased steadily, reaching about 60 p.c. of the original number by 48 hours and 70 p.c. by 72 hours (Table 1).

TABLE 1.

Liver Regeneration in Normal Mice.

Hours after partial hepatectomy	No. of mice used	% of original liver wt. found	% of original no. of nuclei found	Average % of parenchymal cells in mitosis
Series A				
6	3	40·3	36·0	0
12	3	46·6	30·4	0
18	3	49·7	35·4	0
24	5	55·6	31·9	0
30	6	58·4	31·8	0
36	3	65·4	45·0	<1·0
40	7	70·0	51·1	6·7
48	6	70·4	60·3	5·9
72	2	78·8	71·1	2·1
Series B				
48	12	55-70	—	3·4

At 30 hours after partial hepatectomy, only a few mitotic figures were seen. Many more cells in mitosis were seen by 36 hours, but the maximum was reached by 40 hours in series A, the mitotic index then averaging 6·7 p.c. The percentage of mitosis fell considerably after 72 hours. In series B, the mitotic index at 48 hours averaged 3·4 p.c. (Table 1).

The effect of ectromelia virus on the regenerative growth of mouse liver.

The infected hepatectomized mice became obviously unwell 18-20 hours after infection and usually died between 24-30 hours. Infected mice with intact livers rarely sicken before 30 hours and usually die between 36 and 44 hours.

The findings in infected regenerating liver are shown in Table 2. By comparison with Table 1, it is seen that there is a slowing down of cell division following infection, shown both by the lower rate of mitosis, and in series A by the failure of infected liver to reach the same number of cells as the uninfected.

TABLE 2.

Liver Regeneration in Infected Mice.

Hours of intravenous Infection	Hours after partial hepatectomy	No. of mice used	% of original liver weight found (average values)	% of original no. of nuclei (average values)	% of parenchymal cells in mitosis (average values)
Series A					
12	40	13	60·3	41·0	1·4
16	40	4	59·7	—	0·6
23	45	5	60·6	—	0
Series B					
15	48	7	65·7	—	0·5
21	47	5	64·1	—	0·1

Microscopically, the first pathological changes appeared at about 20 hours of infection when commencing nuclear degeneration was seen. This became conspicuous in the later stages of the infection (after 24 hours). The nuclear membrane became wrinkled and finally fragmentation of the nuclear material took place. Crescent-shaped nuclei were also noted. This picture is unlike that seen in the late stages of infection of non-growing livers in which nuclear morphology shows little alteration even when cytoplasmic degeneration appears.

Comparison between regenerative growth of normal and infected livers.

The results are summarized in Table 3. In series A, there is a statistically significant difference between the percentage of original liver weight normally reached by 40 hours after partial hepatectomy and that reached at the same post-operative period in 12-hour infected mice. The percentage of the original liver weight at 40 hours in the infected mice was not much higher than that seen at 30 hours (Table 2) in normal mice. In series B, there seemed to be no difference in restoration of liver mass between normal and infected mice.

In series A, the percentage of original cell number restored by 40 hours of regeneration is less than in the non-infected controls and somewhat higher than non-infected mice at 30 hours (Table 1) of regeneration. This indicates that fewer cells have divided and is in accordance with the mitotic picture found in infected mice at 40 hours (see below). This provides strong evidence that the observed differences in mitosis percentages represent true differences and not different speeds of cell division.

In both series, the percentage of parenchymal cells in mitosis at about 40-50 hours of regeneration is much lower in infected than in normal livers, the difference being statistically significant in all cases. This difference was noted as early as 10 hours of infection (colchicine series) and was striking at 15-20 hours of infection.

TABLE 3.

Comparison between Normal and Infected Regenerating Livers.

(Average values given with standard error.)

Series A (No colchicine)

	No. of mice	% of original liver wt. found	% of parenchymal cells in mitosis	% of original no. of nuclei
40 hr. regeneration Normal mice	7	70·0±0·9	6·7±0·6	51·1
40 hr. regeneration 12 hr. infection	13	60·3±2·1	1·4±0·3	41·0
P values		<·05	<·05	—

Series B (No colchicine)

	No. of mice	% of parenchymal cells in mitosis
48 hr. regeneration Normal mice	7	3·7±1·1
48 hr. regeneration 15 hr. infection	7	0·5±0·4
P values		<·05
47 hr. regeneration Normal mice	5	3·3±0·8
47 hr. regeneration 21 hr. infection	5	0·1±0·06
P values		<·05

Series B (Given colchicine)

	No. of mice	% of parenchymal cells in mitosis
50 hr. regeneration Normal mice	7	5·2±0·6
50 hr. regeneration 10 hr. infection	13	2·1±0·8
P values		<·05
47 hr. regeneration Normal mice	2	8·6±1·4
47 hr. regeneration 20 hr. infection	3	0·0±0·0
P values		<·05

The effect of infection on mitosis cannot be attributed to the injection of liver homogenate for in a separate series (Table 4) it can be seen that no statistically significant difference exists between the mitotic index found in 45-50 hour regenerating livers of mice which had received an injection of normal liver homogenate 16-20 hours prior to sacrifice and that found in the controls. The lower mitosis rate is probably due to the use of older mice in this series.

TABLE 4.

Effect of Normal Homogenate on Hepatic Parenchymal Cell Division.

	No. of mice	% of parenchymal cells in mitosis
45-50 hr. regeneration Untreated mice	14	1·5±0·5
45-50 hr. regeneration 16-20 hr. injection of normal homogenate	14	2·9±0·8
P values		>·05

TABLE 5.

DNA Phosphorus in γ per gram of fresh liver.

Normal non-growing liver	Normal livers 48 hour regeneration	Infected livers (average 17 hours, range 14½-22 hours) 48 hour regeneration
235±44	197±37	189±28

The multiplication of ectromelia virus in regenerating liver.

Fig. 1 shows the results of experiments demonstrating the pattern of ectromelia virus multiplication in regenerating mouse liver, following the injection of 10^9 L.D.$_{50}$ into each mouse. It can be seen that:

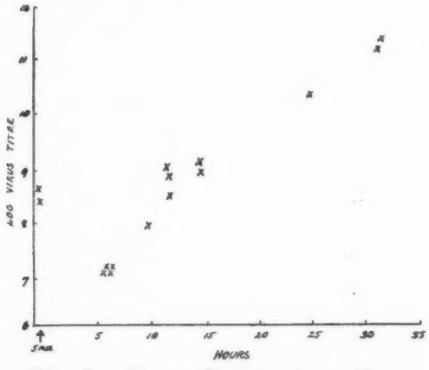

Fig. 1. Hours of intravenous infection. L.D.$_{50}$ titres at various post-operative and post-infective intervals. Seed inoculum in all cases was 10^9 L.D.$_{50}$.

(a) The proportion of virus adsorbed to the liver remnant in 5 minutes is about 1/3 of the seed inoculum.

(b) A fall in infectivity occurs during the next 5-10 hours; the time at which the lowest titre occurs was not established.

(c) The titre between 10 and 12 hours is quite close to the 5-minute value, so that there is then one L.D.$_{50}$ for every one present at 5 minutes.

(d) The titre may possibly remain constant for a short period (12-15 hours).

DNA content of normal livers, normal regenerating and infected regenerating livers.

The results are shown in Table 5. It can be seen that no significant difference exists in the normal and infected 48-hour regenerating livers.

DISCUSSION.

Three findings have resulted from the foregoing experiments: (a) Ectromelia virus tends to inhibit hepatic parenchymal cell division as early as 10 hours after infection, that is towards the end of the eclipse period. (b) The rate of virus multiplication is somewhat faster in regenerating liver than it is in intact liver. (c) The final titre reached in a regenerating liver is as high as the titre found in a non-growing liver.

In intact liver with the same initial multiplicity of infection per cell, Nossal and de Burgh (1954) found the 30-hour titre 10^{10} to $10^{10.4}$ L.D.$_{50}$ per gm., whereas we have found that the titres in regenerating livers at 30 hours are over 10^{11} L.D.$_{50}$/gm. A similar observation has been reported in herpes virus by Modi and Tobin (1954), who have suggested that the multiplication of the virus was more rapid in the proliferating cells of the chorioallantoic membrane than in the normal cells of the membrane.

Inhibition of the onset of mitotic division in the Ehrlich ascites tumour of mice has been noted after infection of the tumour with the Bunyamwera virus (Love and Koprowski, 1953). A significant fall in the number of mitotic figures was accompanied by degenerative changes characterized by the development of prominent pseudonucleoli and the occurrence of multiple nuclear budding. These findings are somewhat similar to the observations reported here, but the inhibition of mitosis was not evident before 24 hours' infection.

The control of mitosis by ectromelia cannot be ascribed to: (a) The effect of the liver fractions, since injection of normal liver homogenate does not affect mitosis rate (Table 4). (b) Failure to regenerate DNA, since the DNA per nucleus is the same in infected and in uninfected livers at the same period of regeneration (Table 5). (c) Pathological changes in the cell, since these only begin to appear after about 20 hours of infection. The control of mitosis is not obviously a secondary change.

At the time when the effect on mitosis becomes evident there are approximately 2 L.D.$_{50}$ per cell, and there is no measurable complement-fixing antigen. The volume of the cell occupied by virus is a minute fraction of the total cell (some 1/25,000 of the cell if one assumes 1 L.D.$_{50}$ to contain 10 virus particles), so the effect would appear to be produced by interference at key centres rather than by action generally throughout the cell.

Bullough (1952) has pointed out that mitosis is very sensitive to alterations in energy supply and it may be that the effect of virus on mitosis is an expression of interference with production or transfer of energy. There is little evidence for interference with production of energy in intact liver for the oxygen uptake, and fructose or pyruvate utilization are normal in the first 24 hours of infection. However, such measurements have not yet been made on infected regenerating livers. Preliminary experiments have shown that there is a fall

in the amount of coenzyme I in both regenerating and infected liver, and a more marked fall in infected regenerating liver. This suggests a mechanism for the more rapid tissue damage found in infected regenerating liver and is a possible mechanism for the alteration in mitosis rate.

SUMMARY.

The effect of ectromelia virus multiplication on the regenerating liver of partially hepatectomized mice has been studied using intravenous inocula of 10^9 $L.D._{50}$ doses. The rate of cell division at a period when maximal rate is normally expected, is less than normal as early as 10 hours after inoculation (that is towards the end of the eclipse period). This effect is even more marked at later periods, mitosis being rare after 20 hours of infection. The rate of virus multiplication in regenerating liver was found to be faster than in intact livers but the maximum titre reached was as high as that found in non-growing livers. The implications of these findings are discussed.

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STUDIES ON INTERMEDIARY IRON METABOLISM

X. THE INFLUENCE OF AGE AND SEX ON THE STORAGE OF SUPPLEMENTAL DIETARY IRON IN THE RAT.

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Under normal conditions, iron absorption from food is determined by what is termed the animal's physiological requirements. However, absorption may be increased under conditions not associated with deficiency of iron, for example, in certain anaemias (Stewart, Vassar and Stone, 1953; Kaldor, 1954), as a result of changes in the chemical composition of food (Finch, Hegsted, Kinney, Thomas, Rath, Haskins, Finch and Fluharty, 1950; Moore and Dubach, 1955), and when the diet is supplemented with large amounts of iron (Finch *et al.*, 1950; Kaldor, 1954 and 1955). Iron absorbed in excess of requirements is deposited in the storage organs, chiefly in the liver and spleen (Finch *et al.*, 1950).

Widdowson and McCance (1948) reported that under normal conditions female rats store considerably more iron in their livers than do males, and that older animals store more than young ones. The purpose of the present work was to investigate the influence, if any, of sex and age on the amount of storage iron in the body of rats when the diet is supplemented with large amounts of iron. It was shown that the rats stored excess iron according to a pattern involving sex and age, but the influence of these factors was not the same as in rats which received a standard diet.

Materials and Methods.

Albino rats of both sexes and of three age groups were used. Each group was further subdivided into those receiving a standard diet and those receiving a diet supplemented with iron. The distribution of the rats is shown in Table 1.

The three age groups represent three distinct phases in the rat's life. At the age of two months it is sexually mature but still growing at a fast rate. At five months it has reached its adult weight and at fifteen months has lived a large part of the average lifetime of a rat and may be showing degenerative changes.

All animals received a standard diet of rat cubes (Animal Health Products, Sydney) and drinking water *ad libitum*. Supplemental iron was supplied by dissolving 1 p.c. ferrous

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[1] This work was undertaken during tenure of a Fellowship from the National Health and Medical Research Council.

TABLE 1.

Distribution of Rats according to Sex and Age.

Age in months	Number of Rats			
	Males		Females	
	Standard diet	Supplemented diet	Standard diet	Supplemented diet
2	7	7	8	7
5	8	10	10	11
15	8	9	9	12

ammonium sulphate in the drinking water. This solution was freshly prepared each day and was acidified with 1 part in 100 of N/10 hydrochloric acid. The rats of the lowest age group were maintained on the iron regimen for a period of two months and those of the two higher age groups for a period of three months. A shorter period was chosen for the younger animals because their capacity to absorb enough iron to show a significant increase in storage was expected to be greater than that of the older animals.

At the end of the test period all rats, including controls, were killed by exsanguination from the heart performed under anaesthesia. Blood was collected for measuring the concentration of haemoglobin by the method of Walsh, Arnold, Lancaster, Coote and Cotter (1953). The livers and spleens were removed and the storage (non-haemoglobin) iron contents measured as described earlier (Kaldor, 1954).

RESULTS.

The mean haemoglobin concentration of heart blood in the different groups is shown graphically in Fig. 1. The male rats on the standard diet had slightly but consistently higher haemoglobin values than the females. The high dietary iron intake apparently did not affect the values in either sex. In three of the six comparisons the iron-supplemented rats had higher values than the control rats, but in the other three the position was reversed. None of these differences proved statistically significant.

The storage iron content of the livers and spleens combined is given in Table 2 and is expressed in absolute amounts and in relation to body weights. The latter expression enables direct comparison between animals of different groups whose size varied considerably. The values for the rats on the standard diet are also shown in a histogram in Fig. 2. The storage iron content of newborn rats was not determined in the present experiment, and a calculated value based on the measurements of Widdowson and McCance (1948) is shown in the Figure. Comparison between males and females demonstrates significant sex differences. It is evident from the histogram that most of the iron stores were laid down during the earlier months. Up to eight months of age the rate of

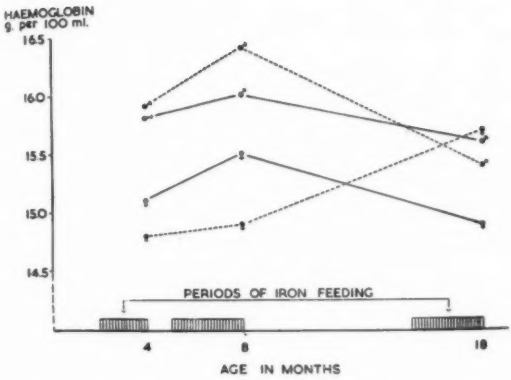

Fig. 1. Mean Haemoglobin Concentration of Heart Blood.
♂ Male rats on standard diet.
♂ Male rats on supplemented diet.
♀ Female rats on standard diet.
♀ Female rats on supplemented diet.

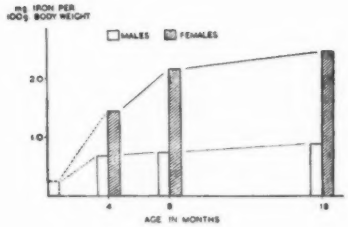

Fig. 2. Storage Iron in Rats on Standard Diet.

storage iron accumulation was greater in females than in males. Between eight and eighteen months very little iron was added to the stores in either sex, and during this period the sex difference in the rate of storage iron accumulation was negligible.

It can be seen from Table 2 that rats which received supplemental iron had considerably larger stores than had rats of the same sex and of comparable age which received the standard diet. The difference between the storage iron of rats on the two diets is the amount of iron accumulated from the supplement added to the standard diet. This quantity is shown in Table 2 for the different age groups of both sexes. It will be recalled that some groups received the supplement for two and others for three months. To enable direct comparison between the groups the total increment found for each was divided by the respective number of months during which the diet was supplemented, and the quantity so computed, termed the monthly increment in storage iron, is shown in the last column of Table 2. The standard error of the monthly increment was obtained by dividing the standard error of the total increment by the appropriate number of months.

Both sex and age appear to influence these results.

1. Females accumulated more of the added iron than did males. The differences were small and almost identical in the three age groups (50, 30 and 40 p.c. respectively). Similar differences could arise by chance in over 5 p.c. of similar trials.

TABLE 2.

Storage Iron of Liver and Spleen Combined.

Sex	Age in months	Rats on standard diet			Rats on supplemented diet					
		Body wt. gm.	Storage iron		Body wt. gm.	Storage iron		Duration of feeding supplemented diet. Months	Increments in storage iron	
			Total mg.	Per 100 gm. body weight mg.		Total mg.	Per 100 gm. body weight mg.		Total	per month
									mg. per 100 gm. body weight	
				*			*		*	*
Males	4	244	1·66	0·68±0·032	255	3·98	1·57±0·095	2	0·88±0·100	0·44±0·050
	8	331	2·45	0·74±0·030	321	6·72	2·09±0·123	3	1·35±0·127	0·45±0·042
	18	337	3·00	0·89±0·086	351	10·01	2·87±0·196	3	1·99±0·214	0·66±0·071
Females	4	171	2·46	1·44±0·034	166	4·64	2·79±0·230	2	1·35±0·232	0·67±0·116
	8	218	4·70	2·16±0·106	220	8·63	3·92±0·155	3	1·76±0·188	0·59±0·063
	18	208	5·12	2·45±0·274	206	10·80	5·25±0·352	3	2·79±0·366	0·93±0·122

* Values shown in this way are the means and the standard errors of the means.

2. In both sexes, rats in the oldest age groups accumulated approximately 50 p.c. more from the added iron than did the younger animals.

A sex difference was found in the distribution of the iron accumulated from the test diet in excess of normal stores. This is shown in Table 3. The figures for the spleen were obtained from the formula:

$$\text{Percentage of excess iron in spleen} = \frac{\text{Iron in spleen of rats fed supplemented diet} - \text{Iron in spleen of rats fed standard diet}}{\text{Iron in liver and spleen of rats fed supplemented diet} - \text{Iron in liver and spleen of rats fed standard diet}}$$

A similar formula was used to obtain figures for the liver. The proportion of excess iron stored in the spleen was approximately twice as high in males as in females in all three age groups. In the rats fed a standard diet, the proportion of storage iron in the spleen was the same in both sexes.

TABLE 3.

Distribution of Iron Accumulated from the Supplemented Diet.

Age in months	Males		Females	
	Percentage		Percentage	
	in liver	in spleen	in liver	in spleen
4	89	11	95	5
8	72	28	85	15
18	72	28	88	12

DISCUSSION.

The results of storage iron assays on rats fed a standard diet demonstrate that females store several times more non-haem iron, weight for weight, than do males. This finding is essentially the same as that reported by Widdowson and McCance (1948). These authors also showed that castration abolishes the sex difference in iron storage in the rat. However, they were unable to find significant sex differences in a number of other vertebrate species. Widdowson and McCance concluded that while sex hormones must play some part in the sex difference of storage iron in rats, there must be other equally important factors involved.

A further analysis was made of the results in the present work. The total iron in the body of the rats which received the standard diet was calculated

from (1) the haemoglobin value and blood volume (6·0 ml. per 100 gm. body weight) and (2) the total storage iron. No allowance was made for the other haem compounds in the body, such as myoglobin and haem enzymes. It was found that the total iron was consistently greater in males than in females, but the iron per 100 gm. body weight was greater in females. This indicates that weight for weight the female requires more iron than the male.

The obvious way in which the female could obtain additional iron is by greater efficiency in absorption from the gastrointestinal tract. As far as is known, excretion of iron from the body is negligible in relation to the total iron content and does not differ in the sexes. The mechanism which regulates absorption of iron from the food must perform a dual function. Firstly, it must ensure the passage of sufficient iron through the mucosal wall to meet physiological requirements. Secondly, it must prevent the absorption of amounts in excess of these requirements. With the range of iron concentrations encountered in a normal diet, the regulating mechanism can apparently function successfully to allow the female rat to absorb a greater percentage of the available iron in the food.

The purpose of feeding a diet containing an excess of iron in the present work was to determine whether females would absorb more of the excess than would males. This was thought possible in view of the sex difference found in rats on a standard diet, and it was found that females did store more of the supplemental iron than did males. However, the difference between the sexes in the rate of iron accumulation was small in all three age groups and was in no instance statistically significant.

Another feature of the storage of excess iron was that both males and females of the oldest age group stored approximately 50 p.c. more than did rats of the same sex in the two lowest age groups. This suggests that the mechanism regulating absorption of iron is less efficient in the older animals in preventing absorption of iron not required by the body. McCay, Lovelace, Sperling, Barnes, Liu, Smith and Saxton (1952) showed that the fluid intake of rats of both sexes diminished significantly with age, irrespective of whether the fluid was water, milk, sucrose solution or coffee. If these findings are applicable to the iron solution used in the present work, it means that the oldest rats received less iron than the younger ones and must have absorbed a considerably greater percentage of that which entered the gastrointestinal tract.

The figures in Table 3 show that the spleen contained a greater percentage of the excess storage iron in males than in females, irrespective of the age of the animals. No adequate explanation can be offered for this observation. It is obvious that the liver cells in the female can store more excess iron than can the liver cells in the male. The determining factors have not been investigated but are possibly hormonal.

SUMMARY.

Storage iron was measured in the livers and spleens of rats of both sexes and of three age groups, fed a standard diet. Females were found to store considerably more iron than males. This sex difference increased progressively during the early months of life but remained constant in adult rats. Old rats had more storage iron than young ones but the rate of iron deposition was higher in young animals than in old ones.

Storage iron was also measured in groups of rats comparable with the above groups but which had received a diet supplemented with iron over a period of months. Storage iron was increased in all groups but the amounts stored per month in excess of the control values did not differ significantly in males and females. The older rats stored a greater excess per month than did the younger animals.

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CARDIAC METABOLISM. IV. SEASONAL VARIATION.

SEASONAL VARIATION IN METABOLISM AND DRUG SENSITIVITY OF THE ISOLATED TOAD HEART (*BUFO MARINUS*)

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(*Accepted for publication 5th December, 1956.*)

It is well established that poikilotherms display seasonal metabolic and physiological variations—see Bullock's review (1955). Whilst some workers have postulated an endocrine basis to explain these modifications (Steir and Taylor, 1939; Carter, 1933; Fromm and Johnson, 1955), there is evidence to suggest that at least some cellular adaptation is involved (Peiss and Field, 1950; Roberts, 1952; Welsh, 1944).

This paper reports experiments to determine whether the isolated toad heart showed any seasonal changes in respiratory quotient values, in glycogen content and in its sensitivity to the cardiac glycoside strophanthin-G.

METHOD.

Male and female toads of the species *Bufo marinus* were used throughout this investigation, the toads being maintained at seasonal temperatures in open cages and fed on a diet of meal worms (*Tenebrio molitor*). Supplies of toads were obtained at approximately monthly intervals thereby reducing the significance of any metabolic disorders due to long periods of captivity. Fresh supplies of toads were allowed at least one week in which to become acclimatized before being used experimentally. Both in their natural environment and in captivity these toads hibernate from early May to late September (Hohnke).

Respiratory Quotient Values.

Throughout the year (January to November) the respiratory quotient values of an extended series of isolated spontaneously beating toad hearts were measured at $25{\cdot}0 \pm 0{\cdot}05°$ C. using the method previously described (Nayler and McKelvie, 1956). The perfusion fluid contained 3 gm. glucose/litre as the sole exogenous substrate. Measurements of oxygen uptake and carbon dioxide output were made at fifteen-minute intervals throughout the four-hour perfusion period; respiratory quotient values were then calculated over half-hourly time intervals.

During March and early April respiratory quotient values were similarly measured (at $25{\cdot}0 \pm 0{\cdot}05°$ C.) in six experiments in which the isolated hearts of non-hibernating toads were perfused with Ringer containing no glucose.

[1] The expenses of this investigation were defrayed by a grant from the Life Insurance Medical Research Fund of Australia and New Zealand.

In July and August the effect of insulin[2] (five units per litre final concentration) upon the respiratory quotient values of the isolated perfused hearts of hibernating toads was measured in six preparations. Normal glucose Ringer was used as the perfusion fluid and all observations made at $25{\cdot}0 \pm 0{\cdot}05°$ C. In a further series of six experiments carried out during August, the respiratory quotient values of the isolated perfused hearts from toads which had been injected two-five days previously with 0·5 gm. anterior pituitary extract[3] were similarly measured. Normal glucose perfusion fluid was used in these preparations and observations made at $25{\cdot}0 \pm 0{\cdot}05°$ C.

Glycogen Determinations.

From February to September inclusive glycogen determinations were made in duplicate on the ventricle tip of freshly pithed toads by the method of Seifter *et al.* (1950). The ventricle tip was removed without delay after pithing the toad so that loss of glycogen due to its breakdown was minimised.

Sensitivity to Strophanthin-G[4].

Using the perfusion apparatus as above, the effect of strophanthin-G (final concentration 1×10^{-5}) on the respiratory quotient values, oxygen uptake, drops per beat, beats per minute, maximum aortic pressure and total work done by the spontaneously beating isolated heart of the hibernating toad was investigated. The results obtained were compared with those reported in a previous communication (Nayler, 1956) in which hearts of non-hibernating toads were similarly used. In both series observations were made at fifteen-minute intervals throughout the four-hour period. In four experiments insulin (5 units per litre final conc.) was added to the perfusate and the response to the glycoside tested as above; in four other experiments the toads were injected intramuscularly with 0·5 gm. anterior pituitary extract 2-5 days before testing. All experiments were performed at $25.0 \pm 0{\cdot}05°$ C.

Toad	Drug	Concentration (final)	Perfusate	No. of Exper.
Non-hibernating	Strophanthin—G	1×10^{-5}	Normal	10
Non-hibernating	Strophanthin—G	1×10^{-6}	Normal	6
Hibernating	Strophanthin—G	1×10^{-5}	Normal	10
Hibernating	Strophanthin—G	4×10^{-5}	Normal	2
Hibernating	Strophanthin—G	1×10^{-5}	+insulin 5 U/litre	4
Hibernating, injected with anterior pit. extract 0·5 gm.	Strophanthin—G	1×10^{-5}	Normal	4

Substrate Substitution.

In view of the apparent loss of sensitivity to strophanthin-G of the isolated hibernating toad's heart perfused with normal glucose Ringer – see results – a series of experiments were performed in which an exogenous substrate other than glucose was used. The alternative substrates used were sodium pyruvate, sodium lactate, sodium citrate, sodium succinate,

[2] Insulin as Insulin B.P. (Boots Pure Drug Co., England).

[3] Anterior pituitary extract (Ambinon) product of Organon Laboratories Ltd. 0·5 gm. injected into gastrocnemius muscle 2-5 days before use.

[4] Strophanthin-G as Ouabain Arnaud : Laboratoire Nativelle product.

TABLE 1.

Respiratory quotient—mean values.[5]

Controls

Time (Min.)	Non-hibernating (6 exp.)	Hibernating (6 exp.)	Hibernating[7] +insulin (4 exp.)	Hibernating[8] +anterior pit. extract (4 exp.)	Non-hibernating No glucose (6 exp.)
0	0·94	0·75	0·72	0·70	0·94
30	0·94	0·75	0·72	0·71	0·95
60	0·94	0·75	0·73	0·72	0·90
90	0·95	0·75	0·73	0·70	0·89
120	0·95	0·75	0·71	0·71	0·89
150	0·95	0·72	0·72	0·72	0·73
180	0·95	0·72	0·72	0·72	0·73
210	0·95	0·72	0·71	0·70	0·73
240	0·94	0·70	0·71	0·70	0·71
Strophanthin—G[6]., 1×10^{-5}					
0	0·95	0·73	0·70	0·72	
30	0·95	0·73	0·71	0·72	
60	0·95	0·72	0·72	0·72	
90	0·95	0·72	0·72	0·71	
120	0·95	0·71	0·71	0·72	
150	0·96	0·71	0·71	0·72	
180	0·96	0·71	0·69	0·72	
210	0·95	0·74	0·69	0·70	
240	0·95	0·73	0·68	0·70	

sodium propionate, sodium glycerophosphate, glycerol and glycine (final concentrations 2 gm./litre).

In this series Straub heart preparations (hibernating) were set up at room temperature using the standard glucose perfusate and the response, if any, to strophanthin-G (1×10^{-5}) recorded on the kymograph. The force of ventricular contraction was taken as an index of the inotropic response. The normal Ringer was then replaced by the modified perfusate (as shown above) and, after ten minutes equilibration, the response to strophanthin-G (1×10^{-5}) was tested. Continuous recordings were made throughout; in each case three experiments were carried out. When sodium salts of exogenous substrates were used the sodium concentration of the perfusate was adjusted to maintain the normal sodium ion concentration.

In a further series of experiments performed in the standard perfusion apparatus the response to strophanthin-G (1×10^{-5}) of the hibernating heart when perfused with modified Ringer containing sodium citrate, sodium pyruvate, sodium lactate or sodium pro-

[5] Respiratory quotient values calculated over thirty minute intervals. Readings taken at fifteen minute intervals.

[6] Strophanthin—G (final concentration 1×10^{-5}) added to the perfusate at the double line (60 mins.).

[7] Insulin-final concentration 5 units/litre.

[8] Anterior pituitary extract (0·5 gm. fresh weight) injected into gastrocnemius muscle 2-5 days previously.

pionate as the exogenous substrate was accurately measured. Each substrate (final conc. 2 gm./litre) was tested in triplicate and the sodium concentration adjusted as before.

The action of strophanthin-G (1×10^{-5}) on the isolated hibernating heart in the presence of

(a) Atropine (4×10^{-5}M) plus acetyl choline (2×10^{-3}M) ..... 6 exp.
(b) Eserine (2×10^{-5}) ..... 3 exp.

was investigated, using the kymograph record of ventricular force of contraction as the index of response.

Throughout this study all dilutions of the drugs used were made in the standard perfusion fluid.

TABLE 2.

Glycogen in ventricle tip expressed in mgm./gm./wet weight.

	Month	Glycogen (mg./gm.)
Non-hibernating	February	14·9
	February	20·7
	February	13·1
	February	10·6
	March	12·9
	March	14·9
		Average = 14·5
Hibernating		
	June	7·5
	June	11·5
	July	7·4
	July	3·5
	July	7·4
	July	11·7
	July	10·3
	August	5·3
	August	9·3
	August	4·1
	August	12·1
	August	7·5
		Average = 8·1

RESULTS.

Respiratory Quotient Values.

Respiratory quotient values of hearts perfused in the standard apparatus at $25{\cdot}0 \pm 0{\cdot}05°$ C. and using the normal Ringer (three gm. glucose per litre) showed marked seasonal variations—see Fig. 1, where the mean results of a large series of experiments are shown.

From October to April values of 0·90-0·95 were consistently recorded; in May the average value fell to 0·85, a minimum of 0·70 being recorded throughout July and August.

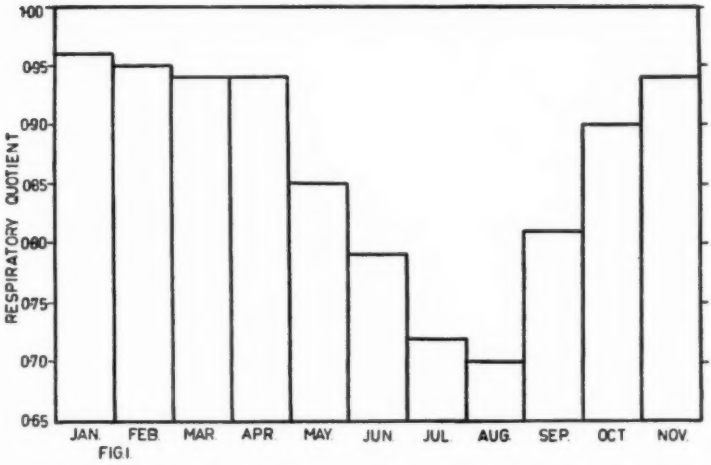

Fig. 1. Respiratory quotient values recorded during initial one-hour perfusion in standard perfusate (mean results of a large series of experiments are shown).

When glucose was deleted from the perfusate of non-hibernating toad hearts and respiratory quotient values noted throughout four hours, a respiratory quotient of 0·93 was recorded during the initial two hours perfusion and subsequently a fall to 0·75 occurred. This depressed value was maintained throughout the remaining time. In Fig. 2 the respiratory quotient values recorded during

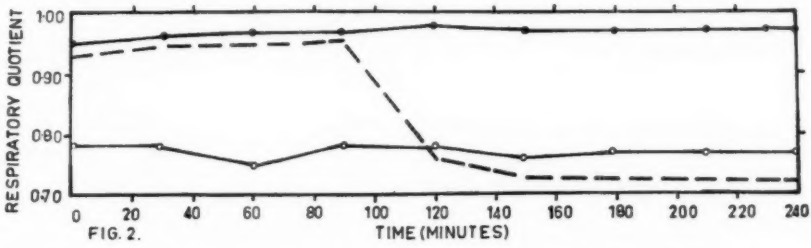

Fig. 2. Respiratory quotient values recorded at thirty-minute intervals throughout four hours perfusion.
Key: Closed dotted line: non-hibernating in glucose Ringer.
Open dotted line: hibernating in glucose Ringer.
Broken line: non-hibernating in non-glucose Ringer.
In each case a typical result of at least six experiments is shown.

the four-hour perfusion of a non-hibernating heart in non-glucose Ringer are compared with those recorded throughout a similar period using hibernating and non-hibernating hearts perfused with standard glucose Ringer.

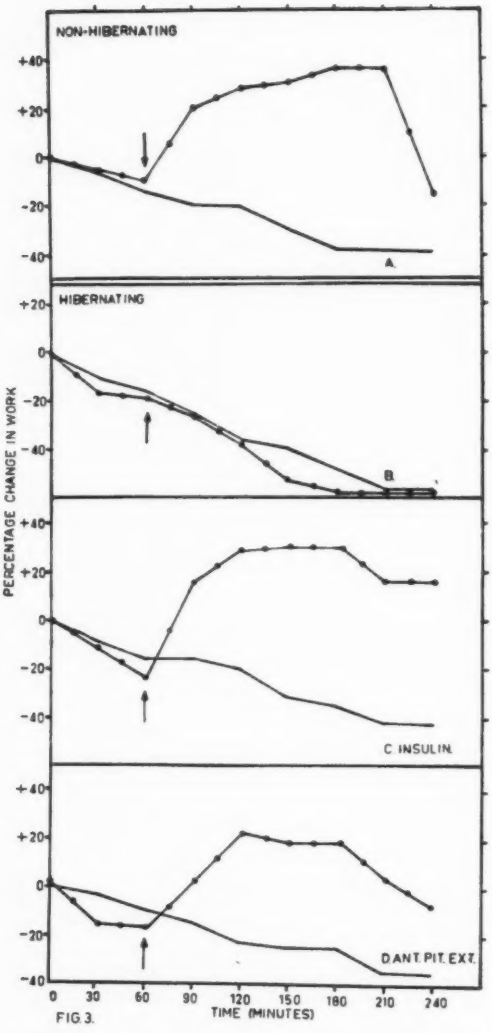

Fig. 3. Percentage change in total work output. Work, in gm. cm./minute measured at fifteen-minute intervals throughout four hours—changes in work output related to initial work capacity of heart.

A. Non-hibernating, standard perfusate: mean results of 6 experiments.

B. Hibernating, standard perfusate: mean results of 6 experiments.

C. Hibernating, insulin 5 U/litre final conc. in standard perfusate: mean results of 4 experiments.

D. Hibernating, previously injected with anterior pituitary extract: mean results of 4 experiments.

Key: Full line—control series.
Dotted line—glycoside treated hearts.

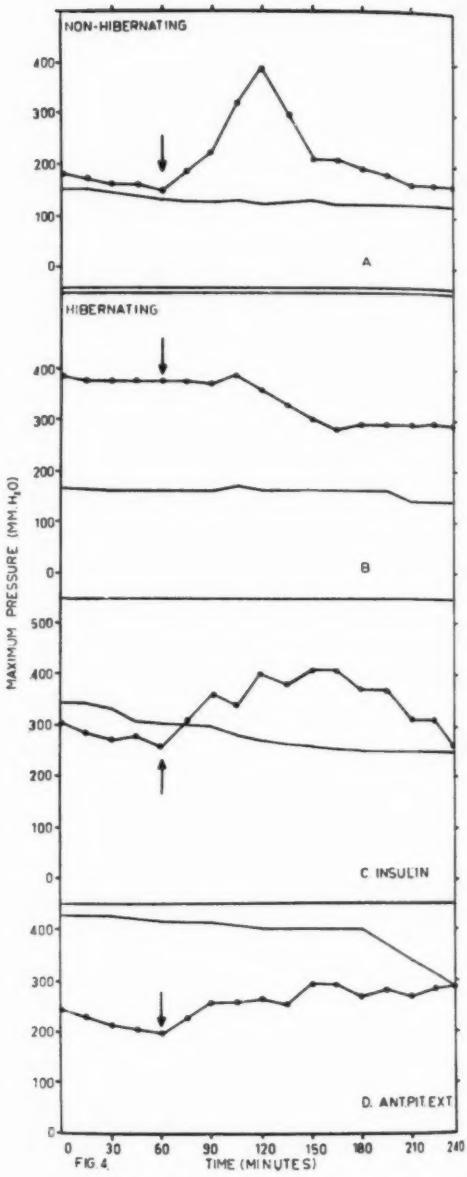

Fig. 4. Maximum aortic pressure measured in mm. of water; strophanthin-G (final conc. 1×10^{-5}) added at the arrow. A, B, C, D, and key as in Fig. 3.

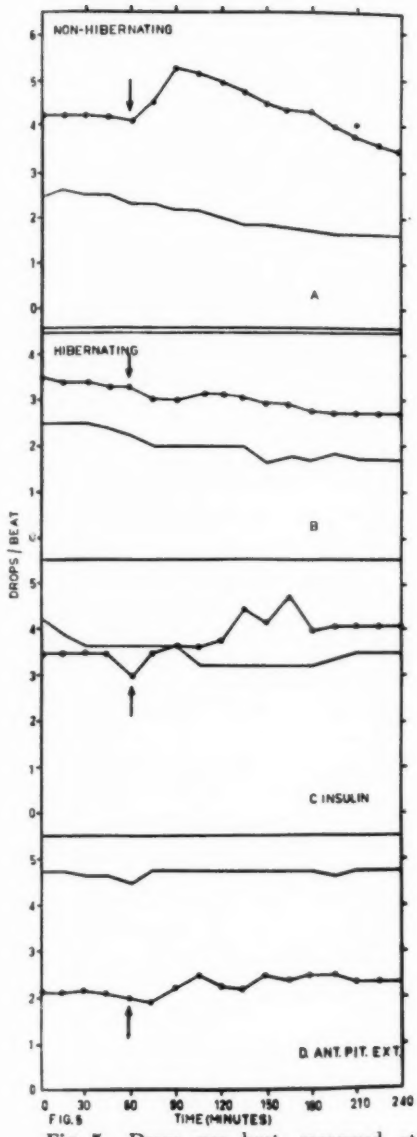

Fig. 5. Drops per beat, measured at fifteen-minute intervals; at arrow strophanthin-G added (final conc. 1×10^{-5}). A, B, C, D, and key as in Fig. 3.

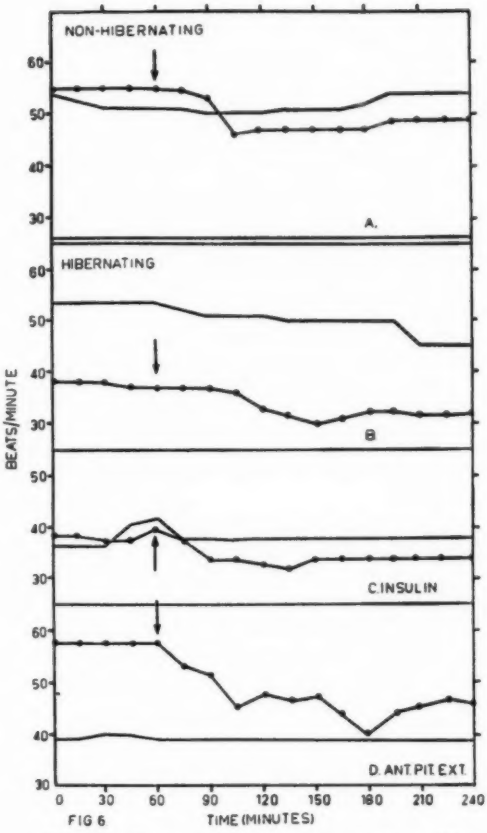

Fig. 6. Beats per minute measured at fifteen-minute intervals; strophanthin-G (final concentration 1×10^{-5}) added at arrow. A, B, C, D and key as in Fig. 3.

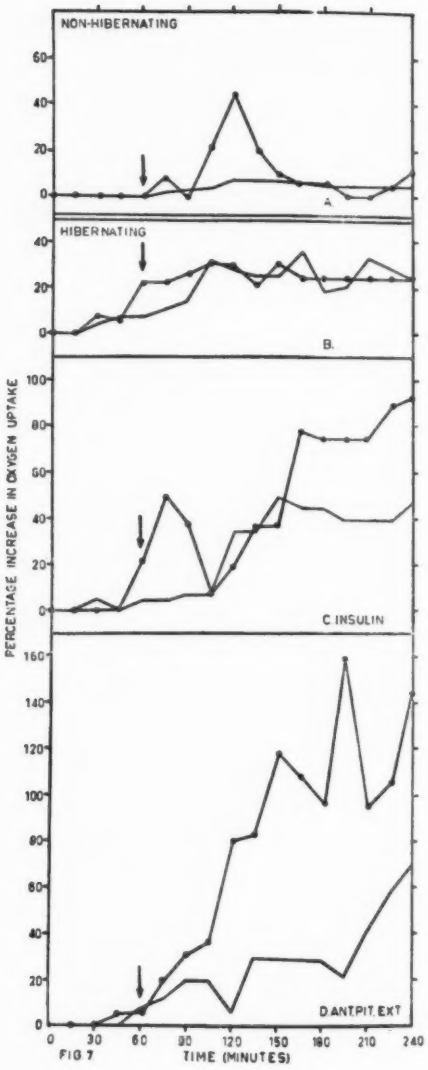

Fig. 7. Oxygen uptake, expressed as the percentage increase; observations made at fifteen-minute intervals throughout four hours perfusion. At arrow, strophanthin-G (final conc. 1×10^{-5} added). A, B, C, D and key as in Fig. 3.

Neither insulin (five units per litre), strophanthin-G (1×10^{-5} and 4×10^{-5}) nor previous intramuscular injections of anterior pituitary extract caused any appreciable change in the low respiratory quotient values noted in the hibernating heart during the four-hour time interval—see Table 1.

Glycogen Content of Ventricle.

As is shown in Table 2, there appeared to be a significant difference in the glycogen content of the ventricle between the hibernating and non-hiberating toads. This variation in glycogen level followed approximately the same time course as did the change in respiratory quotient values observed in Fig. 1. In the present study, glycogen estimations were always made on the ventricle tip, since Davies and Francis (1941) have demonstrated a differential pattern of glycogen distribution throughout the frog's heart.

Sensitivity to Strophanthin-G.

In ten experiments using the standard perfusate an inotropic response was not recorded following the addition of strophanthin-G (1×10^{-5} final concentration) to the hibernating isolated heart—no change in drops per beat, beats per minute, maximum aortic pressure, oxygen uptake or work being observed. In Figs 3, 4, 5, 6, 7, the results obtained above were compared with those reported in a previous study

(Nayler, 1956) in which strophanthin-C in the same concentration, when similarly added to the non-hibernating isolated heart, resulted in a marked inotropic response.

In two experiments the addition of strophanthin-G (final concentration 4×10^{-5}) to the hibernating heart resulted in an inotropic response, qualitatively and quantitatively similar to that previously reported following the administration of strophanthin-G (final conc. 1×10^{-6}) to the non-hibernating heart.

In four experiments the addition of the glycoside (final conc. 1×10^{-5}) to insulin-treated hearts resulted in a small inotropic response. The intramuscular injection of anterior pituitary extract 2-5 days before use likewise resulted in a small inotropic response after the addition of the glycoside; this response was qualitatively identical with that previously reported in non-hibernating tissue. Substrate substitution, the addition of eserine and of atropine all failed to restore the response of the hibernating heart to the glycoside at the above concentration.

During October, the increase in respiratory quotient noted (see Fig. 1) was associated with the gradual return to normal of the heart's response to strophanthin-G.

DISCUSSION.

Welsh (1944) has shown that the response of the isolated heart to some drugs does show variation throughout the year; these investigations, however, have not included the cardiac glycosides. Superficially it might appear that the loss of sensitivity of the isolated heart to strophanthin-G during hibernation, as noted above, was associated with the changed metabolism reflected in the altered respiratory quotient values and reduced glucose utilization (Nayler, unpublished results). However, both insulin and anterior pituitary extract partially restored the response to normal without causing any apparent change in respiratory quotient values. Since exogenous substrates other than glucose did not restore the inotropic response it seems unlikely that the lack of a suitable substrate is involved. Furthermore, as the hibernating heart failed to respond to strophanthin-G in the presence of atropine, eserine or acetyl choline the altered sensitivity reported above does not appear to be due to a changed choline esterase activity.

An alternative explanation is that a change in permeability of the cardiac cell membrane occurs during hibernation, thereby slowing the rate of entry of the glycoside into the cell. Ross (1955) has demonstrated the insulin-like effect of anterior pituitary extract in accelerating the transfer of glucose across the blood aqueous barrier; Fisher and Lindsay (1956), using rat heart, have shown that insulin accelerates the penetration of sugars into the perfused heart. Welsh (1944) was able to modify the response of the winter frog's isolated heart to acetyl choline by the previous administration of insulin, and Keyl and Dragstedt

(1954) were able to modify the response of the embryonic chick heart to several glycosides by the previous administration of insulin.

The results reported here suggest that the previous injection of anterior pituitary extract, and the direct administration of insulin to the isolated heart both either effect a change in cell membrane permeability thus facilitating the entry of the glycoside into the cell, or they play some part in the active absorption of the glycoside.

Reduced respiratory quotient values indicating fat metabolism in hibernating animals have been reported by many workers using intact animals (see Tzukernik, 1938; Kayser, 1940; Fromm and Johnson, 1955; Benedict *et al.*, 1938; and others).

The seasonal changes in respiratory quotient values noted here in the isolated organ indicate the possibility that some enzyme activity in the heart has been modified during hibernation. Nechiporenko (1946) has reported lowered succinic dehydrogenase activity in brain, muscle and tissue of hibernating animals. Since insulin, which is known to accelerate the passage of glucose across a cell membrane, did not restore the respiratory quotient value to the non-hibernating level, it seems unlikely that this apparent disturbance in metabolism is associated with any changed cell membrane permeability occurring during hibernation. The failure of the non-hibernating heart, when perfused with non-glucose Ringer, to show a respiratory quotient pattern similar to that of the hibernating heart supports this view.

It is interesting to note that a significant increase in oxidative metabolism was recorded only in those experiments in which administration of the glycoside to the hibernating heart resulted in an inotropic response. This is in accordance with the theory that increased oxidative metabolism is an integral part of the response of cardiac tissue to the glycosides. Furthermore, these results support Ellis' claim (1953) that the cardiac response to the glycosides is independent of the substrate available, since the inotropic response noted following the addition of strophanthin-G to the fat metabolising heart was qualitatively identical with that previously reported following its administration to carbohydrate metabolising heart (Nayler, 1956).

SUMMARY.

Seasonal variations in the metabolism of the isolated spontaneously beating toad heart have been noted.

These changes included lowered respiratory quotient values and reduced glycogen content.

The hibernating heart showed reduced sensitivity to strophanthin-G; the sensitivity was partially restored to normal by the addition of insulin or the prior injection of anterior pituitary extract.

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THE APPLICATION OF DIFFUSE REFLECTION SPECTROPHOTOMETRY TO CHEMICAL ANALYSIS

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The concentrations of absorbing substances in opaque (i.e. diffusing) material are sometimes required in circumstances that render some of the well-established methods of quantitative analysis impracticable or difficult; this is particularly so with biological material. In some such cases the concentration, or some measure of it, may be found from reflection spectrophotometry using a procedure analogous to the analysis of transparent media by transmission spectrophotometry.

In the example which initiated the present study the amounts of various cytochromes within bacterial cells were required under a wide range of conditions. These cytochromes all possess a common feature, distinctive absorption bands in readily accessible parts of the spectrum. Had the cells been transparent, the simplest method of analysis would undoubtedly have been that of absorption spectrophotometry, Beer's law being used to obtain the relative concentrations in the various samples. At least one absolute measurement by a chemical method would have been required, however, to enable absolute, rather than relative, concentrations to be obtained. But bacterial cells scatter light, and so this method is inapplicable.

In cases of this type spectrochemical analysis must be based on the properties of diffusing media. The laws governing the diffuse transmission and reflection of light by such media are in general very complex and are still imperfectly understood, though the reflecting properties of some types of semi-infinite media (i.e. media so thick as to have negligible transmission) are known exactly. Moreover, the analysis of a medium of finite thickness is in any case much more difficult than that of a semi-infinite medium. Thus we confine attention to a method using the reflection properties of a semi-infinite medium.

Consider a medium containing randomly spaced and randomly oriented scattering particles (the bacterial cells of the above example) dispersed in a matrix which may be air, liquid or solid. Either or both the particles and the matrix may contain various absorbing constituents, the concentrations of one or more of which are to be found. For the time being, suppose that the particles scatter isotropically, the scattering and absorption coefficients of the medium,

σ and a, being defined so that a beam of radiation of intensity I loses amounts $\sigma I ds$ and $aIds$ by scattering and by absorption respectively in a distance ds. The reflectance is then a known function of the ratio a/σ, being independent of the absolute values of a and σ. It follows that a measurement of reflectance is sufficient to yield the ratio a/σ.

Consider now one of the absorbing constituents whose contribution to the absorption coefficient is a_c, the remainder of the absorption coefficient a_b being regarded as "background"; $a_c + a_b = a$. If the reflectance of the medium is measured as a function of wavelength, it will be found to contain a number of reflection minima representing spectral regions where a has maxima, i.e., absorption bands. Some of these correspond to maxima in a_c. We shall show that it is possible to find quantitatively the decrease in reflectance produced by a_c, and so to obtain the value of a_c/σ, and in particular the value corresponding to the centre of the band where the sensitivity is greatest.

The value of a_c/σ is the most that can be obtained using purely spectrophotometric methods. Since a_c is proportional to the concentration of the corresponding absorbing constituent, and σ is proportional to the number of scattering particles per unit volume, a_c/σ is proportional to the concentration of this absorbant per particle. The factor of proportionality, however, depends on

(a) the molecular absorption coefficient for the band; and

(b) the scattering cross section of the particle.

If absolute values are required, (a) necessitates some form of calibration by an independent method. However, (b) is more difficult, as σ is a function of particle shape, size and refractive index, and of wavelength. It is therefore desirable to consider the inherent limitations on the applicability of this method of analysis before describing it in greater detail.

II. Range of Applicability.

In general there are three overriding conditions which must be satisfied:

(i) The particles must be randomly distributed and not clumped together in the form of aggregates; for the relation between reflectance and a/σ varies depending on the degree of aggregation.

(ii) The particles must be well separated as compared with the wavelength of light, or alternatively the particle concentration must be reproduced from sample to sample; for the scattering cross sections of closely packed particles depend on their concentrations, though the magnitude of this effect has not yet been assessed.

(iii) Within a scattering particle, the amounts of substances whose concentrations are required should be small enough and their distributions such that absorption in the particle is proportional to concentration.

In any diffuser there is inevitably a distribution of particle sizes, but we may speak of an appropriate mean size. There are two main classes of medium:

(a) *Mean particle size constant from sample to sample.* Then for a given wavelength the scattering cross section is constant from sample to sample. Measurements of a_c/σ for a given spectral band in different samples yield for each sample the relative concentrations of absorbing substance per particle. The absorbing substance contributing to a_c may be dissolved in the matrix or occur within the scattering particle, but not both; for the relationship between a_c and concentration is different in the two cases.

(b) *Mean particle size variable from sample to sample.* In this case the scattering cross section varies from sample to sample in a way which can best be appreciated by considering scattering by a spherical particle. This has been described mathematically by Mie (1908). While light is scattered symmetrically about the direction of an incident light ray, the intensity of scattered light varies with the angle of scattering θ in a way which depends on the ratio of particle diameter to wavelength. Fig. 1 illustrates the change in angular dis-

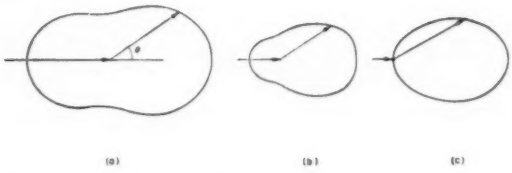

Fig. 1. Relative intensity of radiation scattered through angle θ by spheres of refractive index 1·33 and various radii. Light is incident on the particle from the left, the relative intensity of light scattered through angle θ being indicated by the radius vector. Cases (a), (b) and (c) are for $A = 2\pi r/\lambda = 0{\cdot}5$, 1·0 and 2·0 respectively, where r is the particle radius. To appear on the same scale as case (a), the scales of cases (b) and (c) should be multiplied by 100 and 5000 respectively.

tribution of scattered light as the particle diameter increases. When the diameter is very small compared with the wavelength, scattering is symmetrical in forward and backward directions for normal values of the refractive index, but as the diameter increases, scattering occurs preferentially in the forward direction, with various minima in the angular distribution. At no stage is scattering isotropic. It can be shown, however, that the relation between the reflectance and a/σ for isotropically scattering media also applies fairly closely to semi-infinite media containing spherical scattering particles, provided the actual scattering coefficient σ be replaced by an effective scattering coefficient σ_e, where

$$\sigma_e = \sigma(1-\bar{\mu})$$

and

$$\bar{\mu} = \frac{\int_{-1}^{1} i(\mu)\mu d\mu}{\int_{-1}^{1} i(\mu) d\mu}.$$

Here $\mu = \cos^{-1}\theta$, where θ is the angle of scattering, and $i(\mu)$ is the intensity of light scattered in this direction. On this basis it is possible to express σ_e, for spherical particles in a matrix of refractive index M, in the form

$$\sigma_e = \frac{n\lambda^2}{4\pi M^2} f(m, A),$$

where λ is the wavelength in air, n is the number of particles of radius r per unit volume, m is the ratio of the refractive indices of particle and matrix, and $A = 2\pi Mr/\lambda$ is a measure of the ratio of particle diameter to wavelength. The function $f(m,A)$ can be computed from Mie's results, and is proportional to the particle's effective scattering cross section, at a fixed wavelength, as the diameter varies. On the other hand, we may be interested in the wavelength variation of σ_e for a fixed diameter which, writing σ_e as

$$\sigma_e = \frac{n\pi r^2}{M^2 A^2} f(m, A),$$

is seen to be proportional to $A^{-2}f(m,A)$. Fig. 2 illustrates $f(m,A)$ and $A^{-2}f(m,A)$ for spheres of refractive index 1·33 and 1·55 times that of the surrounding matrix. We note that $f(m,A)$, or the effective scattering cross section, varies much more rapidly than A, at least when A is not too large, but that $A^{-2}f(m,A)$ varies more slowly. Since in most diffusing media the diameters of the scattering particles are greater than or of the order of a wavelength, Fig. 2 indicates that the variation of effective scattering cross section with wavelength is generally slower than inversely with wavelength, and for many purposes can be disregarded.

Thus we find the following conditions for applicability:

(i) Relative concentrations of two or more absorbing substances in the one sample. If these have absorption bands close together, σ can be assumed the same for the centres of the bands; thus the relative concentrations are proportional to the relative values of a_c/σ at the centres of the two bands. The fractional error in this assumption is unlikely to exceed $(\lambda_1 - \lambda_2)/\lambda_1$, where λ_1 and λ_2 are wavelengths of the band centres.

Alternatively, if the band centres are too far separated, and one of the absorbing constituents has a number of absorption bands, the relative values of a_c/σ at the maxima of its absorption bands will vary from sample to sample only because of relative wavelength variations in σ. Thus a plot of the values of a_c/σ against wavelength enables the relative concentrations of two or more absorbing substances to be found. As an extension of this principle, a substance having a number of absorption bands may be deliberately introduced in a known concentration to serve as an internal standard.

(ii) Relative concentration of a substance in different samples. Suppose we require to find the relative concentrations of a substance in two samples, referred to by subscripts a and b. Measurements of the reflectance for the samples yield the results

$$\frac{\alpha_a}{\sigma_a} = X, \frac{\alpha_b}{\sigma_b} = Y.$$

Now make an intimate mixture of equal parts by volume of the two samples, whence the values of the corresponding absorption and scattering coefficients become $\frac{1}{2}(\alpha_a + \alpha_b)$, $\frac{1}{2}(\sigma_a + \sigma_b)$ respectively. The reflectance then yields the result

$$\frac{\alpha_a+\alpha_b}{\sigma_a+\sigma_b} = Z,$$

X, Y and Z being experimentally determined values. Then it follows that

$$\frac{\alpha_a}{\alpha_b} = \frac{Z/Y-1}{1-Z/X},$$

which gives the relative concentrations *per unit volume.*

In the following sections we describe the functional relation between reflectance and α/σ, methods for deriving α_c/σ, and the order of accuracy with which α_c/σ can be obtained.

III. The Spectral Reflectance of a Semi-infinite Diffuser.

Methods for predicting the reflectance of a semi-infinite air-matrix medium have been described by Chandrasekhar (1950), and Giovanelli (1955) has prepared tables of reflectances applicable when the refractive index of the matrix exceeds unity. The reflectance depends on the manner of illumination and observation, though in practice these are likely to be restricted to the cases:

(a) total reflectance (diffuse and specular) for light incident nearly along the normal (as in the G.E. spectrophotometer);

(b) 45° reflectance for light incident along the normal (as in the Beckman, Uvispek and similar spectrophotometers).

Fig. 3 shows the total reflectance R (diffuse and specular) as a function of log α/σ for light incident normally on a semi-infinite isotropically scattering diffuser whose matrix is of refractive index 1·333 (i.e., water), the medium being in contact with a cover glass of refractive index 1·523. Corresponding graphs for other matrices may be prepared from Giovanelli's tables.

In general we may write

$$R = f[\chi] \qquad (3.1)$$

where $\chi = \alpha/\sigma$. It is important to note from Fig. 3 that over a large part of the useful range of the curve the relation between R and log α/σ is fairly linear, so that approximately

$$R = a - c \log_{10} \chi = a - C \log_e \chi \tag{3.2}$$

where

$$C = c \log_e (10).$$

This is equivalent to

$$\chi = K \exp(-R/C) \tag{3.3}$$

where

$$K = \exp(a/C).$$

From Fig. 3, $C \approx 0{\cdot}15$ in the reflectance range $0{\cdot}2$ to $0{\cdot}7$.

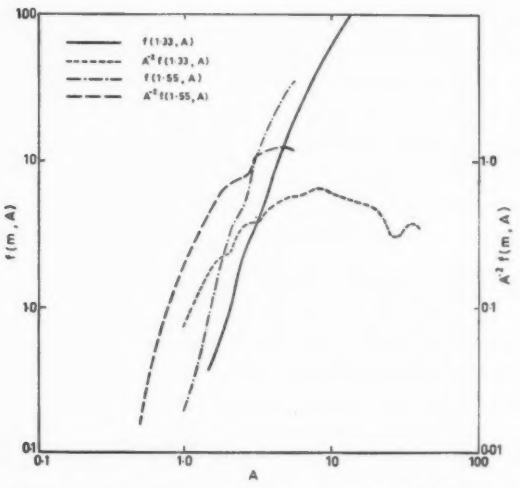

Fig. 2. The functions $f(m,A)$ and $A^{-2}f(m,A)$; both co-ordinates are plotted on a logarithmic scale.

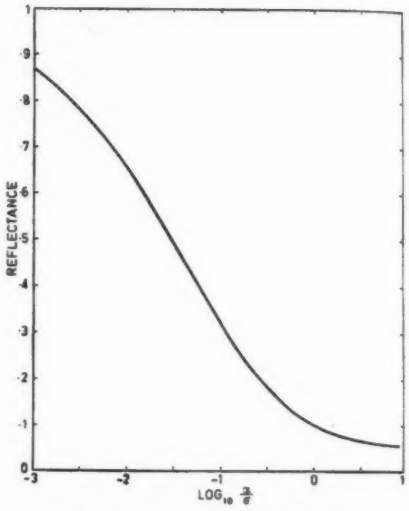

Fig. 3. Total reflectance of a semi-infinite isotropically scattering diffusing medium, the refractive index of the matrix being 1·333. The medium is in contact with a cover glass of refractive index 1·523. Light is incident along the normal to the surface and the total reflectance includes the specular component of magnitude 0·047. The reflectance is plotted as a function of $\log_{10}(\alpha/\sigma)$.

IV. The Background Curve.

(a) *One isolated band.* At any wavelength the absorption coefficient is the sum of the contributions from the various absorbing constituents. Of these constituents, some will be of interest to us; others may be of no special interest and can be regarded merely as providing "background" absorption. For the time being, let us suppose that we are interested in only one absorbing consti-

tuent "A" having only one absorption band centred on wavelength λ_0, where the band absorption coefficient is σ_0. Let the band absorption coefficient elsewhere be a_c, and the background absorption coefficient a_b, so that

$$a = a_b + a_c. \tag{4.1}$$

Corresponding to a_b there will be a "background reflectance"

$$R_b = f[\chi_b] = f[a_b/\sigma],$$

which would be the reflectance of the medium if constituent A were missing.

Under the conditions of §II, the band absorption coefficient will be proportional to concentration k; the coefficient will be a function of wavelength whose value per unit concentration may be written $\phi(\lambda)$, so that

$$a_c = k\phi(\lambda). \tag{4.2}$$

The exact form of $\phi(\lambda)$ may or may not be known, and in the latter case it will often suffice to assume that it takes the form of a damped resonance curve

$$\phi(\lambda) = \frac{g}{(\triangle\lambda)^2 + W^2}, \tag{4.3}$$

where $\triangle\lambda = \lambda - \lambda_0$ is the departure in wavelength from the centre of the band, and g and W are physical constants characteristic of the band, g being related to its strength and W to its width.

Writing $\chi_c = a_c/\sigma$, the difference $\triangle R = R_b - R$ between the actual and background reflectance curves is

$$\begin{aligned} \triangle R &= f[\chi_b] - f[\chi] \\ &= f[\chi - k\phi(\lambda)] - f[\chi]. \end{aligned} \tag{4.4}$$

This relation may be used directly if required, but it will usually be sufficient to take $f[\chi]$ of the form (3·2) whence

$$\begin{aligned} \triangle R &= -C \log_e [\chi_b/\chi] \\ &= -C \log_e [1 - k\phi(\lambda)/a]. \end{aligned} \tag{4.5}$$

From (4·3), this may be written

$$\triangle R = -C \log_e\left[1 - \frac{kg}{a\{(\triangle\lambda)^2 + W^2\}}\right] = -C \log_e\left[1 - \frac{kg}{aW^2\{1 + (\triangle\lambda/W)^2\}}\right]. \tag{4.6}$$

In the special case where R/C is small,

$$\triangle R = \frac{Ckg}{aW^2\{1 + (\triangle\lambda/W)^2\}}; \tag{4.7}$$

since $C \approx 0{\cdot}15$ in the central reflectance range in Fig. 3, equation (4·7) is only valid when $R \lesssim 0{\cdot}04$. It may be written as

$$\frac{\triangle R}{\triangle R_0} = \frac{1}{1 + (\triangle\lambda/W)^2}, \tag{4.8}$$

where $\triangle R_0$ is the value of $\triangle R$ at the centre of the band ($\triangle\lambda = 0$) and W turns

out to be the value of $\triangle\lambda$ for which $\triangle R = \frac{1}{2}\triangle R_0$. In this form the shapes of all weak absorption bands obeying (4·3) are identical.

(b) *Several widely spaced bands.* It will be seen from (4·3) and (4·8) that the wings of an absorption band cover the full spectrum, though its effects will be negligible far enough from the band centre. In fitting a background curve when there are several bands of interest, it is necessary to interpolate graphically between those parts of the spectral reflectance curve which are little affected by the presence of the absorption bands. These are readily found if there are only widely spaced bands of known half-width W, for the various $\triangle R_0$ may be estimated and the appropriate values of $\triangle\lambda$ found beyond which the curve is unaffected (i.e., $\triangle R$ falls below the limits of measurement). The background curve is then drawn and the final values of $\triangle R_0$ obtained. If an absorption band is shallow, (4·8) may be used for this part of the work. For a strong band, (4·5) may be rewritten as

$$\frac{kg}{aW^2} = 1 - exp(\triangle R_0/C),$$

so that in the wings of the band where $\triangle R$ is small,

$$\triangle R = \frac{C[exp(\triangle R_0/C) - 1]}{1 + (\triangle\lambda/W)^2}. \qquad (4.9)$$

While in (4·9) W is still the same physical constant as in (4·8), it is no longer, for a deep band, the value of $\triangle\lambda$ for which $\triangle R = \frac{1}{2}\triangle R_0$.

When W is initially unknown, its value must first be estimated so that those parts of the background which are unaffected can be ascertained: when the background spectral reflectance curve is drawn, the value of W readily follows, and a check should be made that the unaffected background has been selected validly.

(c) *Overlapping bands.* In the presence of overlapping absorption bands the establishment of the background presents greater difficulty but it can usually be found with adequate precision. It is first necessary to estimate the various values of $\triangle R_0$ at the centres of each band, and to estimate the various values of W also, if unknown. From these estimates, the contributions of each band to the difference between the actual and background reflectance curves may be found from (4·8), or (4·9) if necessary, for a sufficient number of wavelengths where the difference is small, e.g., at maxima of the spectral reflectance curve. From these an improved background curve may be obtained. Further, contributions from each other band to the difference between curve and background must be computed for the centres of each band, λ_0, and for the corresponding wavelengths $\lambda_0 \pm W$. From these, the new values of $\triangle R_0$ and W may then be obtained. If W is known, e.g. from analysis of a previous sample, the computation is greatly facilitated; thus, making the initial assumption that the contribu-

tion from all other bands at the centre of a given band, λ_0, is the mean of the contributions at $\lambda_0 \pm W$, the height of the straight line joining the points at $\lambda_0 \pm W$ above the curve at λ_0 is $\frac{1}{2}\triangle R_0$.

For all the above computations a slide rule gives greatest speed and sufficient accuracy.

To illustrate the procedure we consider fitting the background to the spectral reflectance curve of a suspension of *Aerobacter aerogenes* containing cytochromes. The spectral reflectance curve, obtained on a G.E. spectrophotometer, is shown in Fig. 4 and various strong and weak bands may be noted, centred on wavelengths 426, 484, 529, 560, 598 and 633 mμ respectively.

The initially estimated position of the background curve is shown by a broken line in Fig. 4, and from this the first estimates of $\triangle R_0$ and W have been made, as listed in Table 1 under 1st Approximation: these estimates include allowances for overlapping bands. The strength of the 426 mμ band is such that its shape can be described only by (4·5), though (4·9) may be used in the wings. For the other bands (4·8) is adequate. Using these relations and the initial estimates of $\triangle R_0$ and W, the strengths of each band have been found in the regions of the maxima and "half-width" points, λ_0 and $\lambda_0 \pm W$, of each other band. An extract from these computations relating to the estimated strengths of the various other bands near the maximum of the 529 mμ band is shown in Table 2. The estimated contributions from each band at the reflectance maxima 507, 543, 582 and 610 mμ, and at the ends of the curve 400 and 700 mμ, are found in the same way, and the totals for each wavelength added to the observed reflectances to obtain points on the second approximation to the background curve.

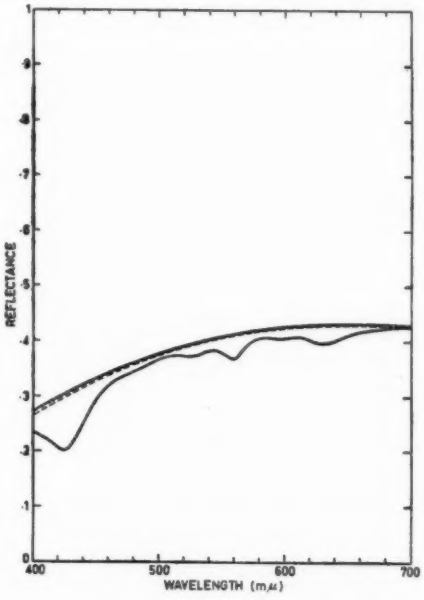

Fig. 4. Spectral reflectance (lower curve) of *Aerobacter aerogenes* showing the Sorèt cytochrome absorption band centred on 426 mμ, the α and β bands of cytochrome b_1 at 529 and 560 mμ, a band at 598 mμ due to cytochrome a_1, and at 633 due to cytochrome a_2. A weak band of unknown origin also appears centred on 484 mμ. The dotted curve is the initial estimate of the background; the upper continuous curve is the final background when the effects of cytochrome absorption bands have been removed.

TABLE 1.

Successive approximations to the various R_0 and W in Fig. 4.

Band (mμ)	Approximation											
	1st		2nd				3rd					
	$\triangle R_0 \times 10^2$	W	$\triangle R_0 \times 10^2$			W	$\triangle R_0 \times 10^2$			W		
426	9·8	16	10·3	−0·0	= 10·3	16	10·3	−0·0	= 10·3	16		
484	0·6	6	—			—	—			—		
529	1·5	10	2·2	0·7	1·5	11	2·2	0·9	1·3	11		
560	3·5	10	4·1	0·5	3·6	10	4·4	0·7	3·7	10		
598	0·6	6	1·8	1·1	0·7	14	2·0	1·3	0·7	13		
633	3·5	19	3·2	0·2	3·0	22	3·5	0·3	3·2	22		

TABLE 2.

Strengths of the wings of the various bands near the centre of the 529 mμ band in Fig. 4.

Band (mμ)	Strength of wing at wavelength indicated ($\triangle R \times 10^2$)								
	Approximation								
	1st			2nd			3rd		
	519	529	539	519	529	539	519	529	539
426	0·4	0·3	0·3	0·4	0·35	0·3	0·4	0·35	0·3
484	—	—	—	—	—	—	—	—	—
560	0·2	0·3	0·7	0·2	0·35	0·65	0·2	0·35	0·65
598	—	—	—	—	0·05	0·05	—	—	—
633	0·1	0·1	0·1	0·1	0·15	0·2	0·1	0·15	0·2
Total	0·7	0·7	1·1	0·7	0·9	1·2	0·7	0·85	1·15

Under the heading 2nd Approx. in Table 1 we list now the value of $\triangle R_0$ read from the graph, from which is then substracted the total estimated strength of other bands at this wavelength (given by the first approximation in Table 2 for 529 mμ), to yield the second approximation to $\triangle R_0$. For each band the second approximation to W is obtained after correcting for the total estimated strengths of the wings of the other bands near the points $\lambda_0 \pm W$. The strength of the wings of the 426 mμ band is derived from (4·5), which may be rewritten for this purpose as

$$\triangle R = C \log_e \left[1 - \frac{1 - exp(\triangle R_0 / C)}{1 + (\triangle \lambda / W)^2} \right].$$

For the wings of the other bands (4·8) is used.

The upper continuous curve in Fig. 4 shows the final background yielded by this process of iteration.

Finally, the values of $(k/\sigma)\phi(\lambda_0)$, which provide the required measures of the concentrations of the corresponding constituents, are obtained from the relation

$$\frac{k}{\sigma}\phi(\lambda_0)\frac{a_c}{\sigma} = \chi - \chi_1 \tag{4.10}$$

where χ is read from Fig. 3 corresponding to R_0, and χ_1 corresponds to $R_0 + \triangle R_0$. From (3·3), (4·10) reduces to

$$\frac{k}{\sigma}\phi(\lambda_0) = \chi[1 - exp(-\triangle R_0/C)]. \tag{4.11}$$

This completes the analysis of the spectral reflectance curve, and yields the relative concentrations under conditions listed in § II.

V. Accuracy.

(a) Sensitivity. The sensitivity of the method may be found from (3·2) from which, if C is known exactly

$$\frac{da}{a} = -\frac{dR}{C}. \tag{5.1}$$

Since $C \approx 0{\cdot}15$,

$$\frac{da}{a} \approx -7dR. \tag{5.2}$$

The best accuracy normally obtainable in a measurement of R is about $\pm 0{\cdot}001$, so that the highest sensitivity obtainable in measuring a or a/σ is about $\pm 0{\cdot}7$ p.c. Writing $a = a_b + k\phi(\lambda)$, the smallest detectable change in concentration k is given by

$$\frac{\triangle k}{k} = \frac{\{(\triangle a)^2 + (\triangle a_b)^2\}^{\frac{1}{2}}}{a - a_b}. \tag{5.3}$$

At best, the uncertainty in the background absorption co-efficient will be similar to (5·2), whence

$$\frac{\triangle k}{k} \approx \frac{7\triangle R(a^2 + a_b^2)^{\frac{1}{2}}}{a - a_b}. \tag{5.4}$$

When $a_b << a$, the sensitivity is almost independent of the background values, but when a-a_b is very small, the sensitivity is poor.

(b) Effect of uncertainties in the curve of R against a/σ. In general it is unlikely that the nature of the scattering will be known, so that the appropriate curve of R as a function of a/σ will be uncertain. Theoretical studies of curves obtained with different modes of scattering have indicated that the constant C in (3·2) is unlikely to vary, from this cause, by more than ten per cent.

The effect of an incorrect value of C may be estimated as follows: let

$$\chi_1 = \frac{a-a_b}{\sigma} = K[exp(-R_1/C_1) - exp(-R_{b1}/C_1)],$$

where the subscript unity refers to measurements made on a given material. For a second material with a different background curve,

$$\chi_2 = K[exp(-R_2/C_2) - exp(-R_{b2}/C_2)].$$

The ratio κ of the concentrations is given by

$$\kappa = \frac{\chi_1}{\chi_2} = \frac{exp(-R_1/C_1) - exp(-R_{b1}/C_1)}{exp(-R_2/C_2) - exp(-R_{b2}/C_2)}. \tag{5.5}$$

There are two special cases:

(i) Background absorption negligible. Then (5·5) is replaced by

$$\kappa = exp[-R_1/C_1 + R_2/C_2]$$

and the fractional error in the estimated ratio of concentrations due to an error $\triangle C$ in C is

$$\frac{\triangle\kappa}{\kappa} = \frac{R_1\triangle C_1}{C_1^2} - \frac{R_2\triangle C_2}{C_2^2}. \tag{5.6}$$

If it is known that the scattering particles are similar in the two materials, so that C_1 and C_2 may be assumed identical, (5·6) simplifies to

$$\frac{\triangle\kappa}{\kappa} = \frac{R_1-R_2}{C} \cdot \frac{\triangle C}{C}.$$

At worst, $\triangle C/C \approx 0{\cdot}1$. Since $C \approx 0{\cdot}15$,

$$\frac{\triangle\kappa}{\kappa} \lesssim 0.7(R_1-R_2) \tag{5.7}$$

and vanishes if $R_1 = R_2$.

(ii) Weak absorption bands. If $C_1 = C_2 = C$ here also, then

$$\kappa = \frac{R_{b1}-R_1}{R_{b2}-R_2} exp[-(R_1-R_2)/C]$$
$$= \frac{\triangle R_{01}}{\triangle R_{02}} exp[-(R_1-R_2)/C],$$

from which

$$\frac{\triangle\kappa}{\kappa} \approx \frac{R_1-R_2}{C} \cdot \frac{\triangle C}{C} \lesssim 0.7(R_1-R_2) \tag{5.8}$$

as before.

It is clear that even if the exact law of reflectance is unknown high precision can still be obtained if the reflectances of the various media do not differ greatly.

VI. Depths of the Various Reflection Bands of the One Substance.

The strengths of the various reflection bands of a substance are not necessarily proportional to the corresponding band absorption coefficients. To see this, we note that at the centre of a band (4·6) can also be written as

$$R = C \log_e (1 + k\alpha'/\alpha_b)$$

where $\alpha' = g/\alpha W^2$. For simplicity, suppose R is small, so

$$R = Ck\alpha'/\alpha_b.$$

Now consider two spectral bands, distinguished by subscripts 1 and 2; the relative strengths of the reflection bands are then

$$\frac{\triangle R_1}{\triangle R_2} = \frac{\alpha_1{}^1}{\alpha_2{}^1} \cdot \frac{\alpha_{b2}}{\alpha_{b1}}.$$

The significant point is that the ratio α_{b2}/α_{b1} may be very different from unity. Thus in Fig. 4, for the two bands at 426 and 560 mμ, denoted by subscripts 1 and 2 respectively, the background reflectances are 0·30 and 0·42, so that, from Fig. 3, we find

$$\alpha_{b2}/\alpha_{b1} = 0.46\, \sigma_2/\sigma_1.$$

The ratio σ_2/σ_1 is unknown, but the scattering coefficient increases as the wavelength decreases, so $\sigma_2/\sigma_1 < 1$. A lower limit to σ_2/σ_1 is given by the laws of Rayleigh scattering, so that

$$1 > \sigma_2/\sigma_1 > (\lambda_1/\lambda_1)^4 = 0.34.$$

Assuming an intermediate value, $\sigma_2/\sigma_1 \approx 0{\cdot}75$,

$$\frac{\triangle R_1}{\triangle R_2} \approx 0.35\, \alpha'_1/\alpha'_2.$$

In other words, in this case the ratio of the depths of the reflection bands is only about one-third that of the absorption coefficients.

SUMMARY.

This paper deals with the quantitative analysis of diffusing media using a technique of diffuse reflection spectrophotometry. Spectral reflectance measurements on a thick enough layer of medium enable the ratio of absorption and scattering coefficients, α and σ, to be obtained. The various absorbing constituents each have one or more absorption bands which give rise to minima in spectral reflectance.

Methods are described for finding the various α_c/σ, α_c being the contribution of any constituent to the absorption coefficient at the centre of its absorption band. Since σ usually varies only slowly with wavelengths, the α_c/σ yield relative concentrations of the different absorbing components in a given sample;

methods are also available for obtaining relative concentrations in different samples, depending on whether σ is constant or variable from sample to sample.

Acknowledgment. An appreciation of the need for a technique of quantitative diffuse reflection spectrophotometry arose out of discussions with Dr. F. Moss, who also supplied the sample whose spectral reflectance is shown in Fig. 4.

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A COMPARISON OF METHODS FOR THE ISOLATION OF MELANOCYTE STIMULATING HORMONE FROM BLOOD

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Melanocyte stimulating activity in human blood and urine has been reported sporadically over the past thirty years. Several workers (Konsuloff, 1934; Mandelstamm, 1935; and Jores, 1936) claimed that the presence of this hormone in the urine was a useful test for pregnancy, although Landgrebe and Waring (1941) concluded that the darkening of Xenopus toads produced by urine extracts was not due to melanocyte stimulating hormone (MSH). Jores (1933) reported that an extract of an acetone precipitate of blood with dilute acetic acid and alcohol caused darkening of isolated frog skin, and claimed that this was due to MSH. Cunningham-Dax (1938) made similar extracts from the blood of patients suffering from retinitis pigmentosa and reported that increased amounts of MSH were present. These results have not been confirmed, nor were Landgrebe and Waring (1941) able to repeat Jores' experiments. Using living animals for assay they were not able to detect melanocyte stimulating activity in blood and using Jores' extraction procedure, recovered, at the best, only 60 p.c. of added hormone.

More recently, the isolation of corticotropin (ACTH) from plasma has been described by Bornstein and Trewhella (1950) and confirmed by Parrott (1955). This method relies upon acid-acetone extraction of blood or plasma and yields substantial recoveries of ACTH. Sulman (1952) has applied a modification of this method to the extraction of MSH from the blood. Johnsson and Högberg (1953) have used phosphotungstic acid precipitation and claim to have shown excess melanocyte stimulating activity in the blood of patients suffering from Addison's disease. Sydnor and Sayers (1952) have described a method for the recovery of ACTH from blood by glacial acetic acid extraction and subsequent concentration on oxycellulose.

In the course of a series of studies on the relationship of MSH to certain clinical disorders of pigmentation, we have had reason to examine some of these

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reported methods in detail. The purpose of this paper is to report briefly on the efficiency of different methods for the recovery of this hormone from the blood, and to describe a modified method for this purpose.

MATERIALS AND METHODS.

Assay Procedure.

The method of assay has been described (Hudson and Bentley, 1957). This method involved the use of normal, light-adapted male Xenopus laevis toads. In each instance sixteen toads were used, with four toads at each of two dose levels of standard and unknown. The MSH used in all these experiments was that contained in Commonwealth Serum Laboratories Corticotropin (Batch P-60). Our usual procedure was to add known amounts (250-1000 μg.) of this hormone to samples of blood from normal persons, the MSH content of which was negligible, and to adjust the final volume of extract to give MSH concentrations of the same order as the standard on the assumption of 75 p.c. recovery.

Methods of Extraction.

The following methods have been investigated:

(1) *Sulman's Method* involves the precipitation of red cells and proteins and extraction of MSH by acid-acetone. The hormone is then precipitated from the supernatant by ether, washed and dried with ether and dissolved in weak alkali. After neutralisation this solution is injected into the test animal.

(2) *Johnsson and Högberg's Method* uses serum, from which the proteins are precipitated by 20 p.c. trichloracetic acid. The supernatant is treated with 5N H_2SO_4, the MSH precipitated by saturated phosphotungstic acid and dissolved in 0·1N NaOH. Phosphotungstic acid is removed by the anion exchange resin Amberlite, IRA-400.

(3) *Method of Sydnor and Sayers.* The Astwood technique for concentrating ACTH is utilised. Blood is taken into four volumes of glacial acetic acid and stirred for thirty minutes at 70-75° C. After dilution and treatment with Hyflo Supercel, the acid extract is stirred with oxycellulose for 24 hours. The oxycellulose is collected and washed with 0·1N acetic acid. MSH is eluted from the oxycellulose by 0·1N HCl.

(4) *Method currently used.* This employs acid-acetone or acetone extraction and oxycellulose concentration of MSH. Venous blood, to which a known amount of MSH has been added, is mixed immediately with an equal volume of acetone and stirred for 2 minutes. After centrifuging, the supernatant is collected and the precipitate treated with a similar volume of 50 p.c. aqueous acetone. This is stirred for 2 minutes and then centrifuged.

The acetone from the combined supernatants is removed under reduced pressure at 40° C. The volume of the remaining aqueous extract is measured, and sufficient glacial acetic acid added to make the solution approximately 0·1N with respect to acetic acid. To this solution is added 200-400 mg. of oxycellulose suitably prepared by the method described by Sydnor and Sayers (1952). (Oxycellulose with 10-12 p.c. carboxyl groups, obtained from the Tennessee Eastman Company, is washed three times with 100 ml. of 0·1N HCl, twice with 100 ml. distilled water and finally with 100 ml. 0·1N acetic acid twice.) This mixture is placed on a revolving drum for 24 hours to permit complete adsorption of the MSH on the oxycellulose. The oxycellulose is then collected on a coarse sintered glass funnel and washed with 50-100 ml. 0·1N acetic acid. It is then suspended in an appropriate volume of 0·1N HCl (usually less than 5 ml.), and mixed on a revolving drum for at least one hour to permit elution of the MSH from the oxycellulose. The eluate is collected by filtration, neutralised with NaOH (bromthymol blue paper) and made up to a suitable volume with saline solution so that the final tonicity is approximately the same as that of the Ringer's solution described by Landgrebe and Waring (1944). In this state it is suitable for injection into Xenopus laevis toads.

Prior to the investigation of any methods which involved the concentration of MSH on oxycellulose, a series of experiments was performed to investigate the recovery of hormone from oxycellulose. Known amounts of hormone were added to oxycellulose in 0·1N acetic acid, a period of 24 hours being permitted for adsorption. At the end of this time the oxycellulose was collected on a coarse sintered glass funnel and washed with 50-100 ml. of 0·1N acetic acid. The hormone was then eluted from the oxycellulose with 0·1N HCl. and assayed by using intact Xenopus toads as has been described (Hudson and Bentley, 1957).

RESULTS.

Recovery from oxycellulose.

Using the method outlined, the recovery of added MSH from oxycellulose varied between 93-98 p.c. in a series of three experiments. The results are shown in Table 1.

TABLE 1.

Showing recovery of MSH from varying amounts of oxycellulose (adsorption in 0·1N acetic acid and elution with 0·1N HCl).

Amount of added hormone (μg.)	Amount of oxycellulose (mg.)	Recovery p.c.
250	25	98
250	50	93
250	100	98

Sulman's Method.

The addition of aqueous solutions containing 500 μg. of our reference powder to plasma yielded recoveries of between 23 and 58 p.c. in a series of four experiments. When the hormone was added to whole blood the recovery was between 25 and 36 p.c. Addition of the hormone to whole blood and the subsequent extraction of the serum gave practically zero recovery in two experiments.

Phosphotungstic acid precipitation.

Inspection of the results from Table 2 will show that recovery of MSH from blood or plasma by this method was uniformly low, the values ranging from 10-20 p.c. The treatment of the serum from blood to which our reference material had been added gave virtually no recovery.

Method of Sydnor and Sayers.

The recovery of MSH when this hormone was added to whole blood or plasma ranged between 25 and 76 p.c. in a series of five experiments. Addition of hormone to blood and extraction of the serum yielded less than 5 p.c. recovery.

Acetone extraction: oxycellulose adsorption.

When the reference material was added to whole blood or plasma and extracted immediately, recoveries ranged between 55 and 84 p.c. It is apparent from Table 2 that the use of acid-acetone in the initial extraction and precipitation did not materially improve recovery; if anything, better recoveries were obtained than when no acid was added to the acetone. It will be noted that in those instances when the hormone was added to whole blood and the plasma or serum of that sample was extracted, there was conspicuously low recovery. The results of these different methods are shown in Table 2.

TABLE 2.

Showing the recoveries of added amounts of MSH to blood, plasma and serum yielded by different methods.

MSH added to:	MSH recovered from:	MSH added: (μg.)	p.c. recovery	Method
Plasma	Plasma	500	23	
Plasma	Plasma	500	58	
Plasma	Plasma	500	45	Sulman (1952)
Plasma	Plasma	500	39	
Blood	Blood	500	25	Acetone extraction, ether precipitation.
Blood	Blood	500	36	
Blood	Plasma	500	<5	
Blood	Serum	500	<5	
Blood	Serum	1000	<5	
Blood	Serum	500	<5	
Blood	Serum	1000	<5	Johnsson and Högberg (1953)
Blood	Blood	250	12	
Blood	Blood	500	20	Phosphotungstic acid precipitation.
Plasma	Plasma	500	10	
Blood	Blood	500	25	
Blood	Blood	500	45	
Blood	Blood	500	56	Sydnor and Sayers (1952)
Blood	Blood	250	76	
Blood	Blood	250	68	Acetic acid extraction oxycellulose concentration.
Blood	Plasma	500	20	
Blood	Serum	500	<5	
Plasma	Plasma	500	60	
Blood	Blood	500	63*	
Blood	Blood	500	71*	
Blood	Blood	500	83	Currently described.
Blood	Blood	500	78	Acetone extraction, oxycellulose concentration.
Blood	Blood	500	84	
Plasma	Plasma	500	55	
Plasma	Plasma	500	80	*Acid-acetone extraction.
Plasma	Plasma	500	62	
Serum	Serum	500	70	
Blood	Plasma	500	10	
Blood	Plasma	500	15	
Blood	Serum	500	<5	

DISCUSSION.

These results leave little doubt that the most satisfactory method for the recovery of MSH from blood or plasma is one that involves concentration of the hormone by an oxycellulose technique. It would seem that the method we have developed gives consistently higher recoveries of added hormone than that described by Sydnor and Sayers. Although they describe the recoveries of corticotropin, there is good reason to believe that both substances behave similarly with respect to adsorption and elution from oxycellulose. In three experiments we have been able to recover between 85-100 p.c. of the melanocyte stimulating activity from oxycellulose, indicating that slight losses occur at this stage. The poor recoveries obtained from other methods indicate that their use in experiments on corticotropic or melanocyte stimulating activity of blood or plasma is unreliable, and results of such experiments must be accepted as such. Although we repeated the technique described by Johnsson and Högberg in every detail, we were never able to obtain high recoveries of added hormone.

Another consistent finding in this series of experiments was the conspicuous loss of MSH when attempts were made to recover this from plasma or serum after the hormone had been added to fresh whole blood. Sydnor and Sayers (1953) have shown, with respect to corticotropin, that this is not due to inactivation by the freshly withdrawn blood. If this is true for MSH, our results suggest that this loss may be due to adsorption of the hormone on to red blood cells. It is of interest to note that all the results reported by Johnsson and Högberg in which they claim to have detected excessive amounts of MSH in a variety of clinical conditions (Addison's disease, adrenalectomy, myocardial infarction, etc.), were obtained from the serum rather than from fresh whole blood. The results of these present experiments are not compatible with these findings.

SUMMARY.

The methods for the isolation of MSH from blood have been reviewed. A modified method has been described for the isolation of this hormone from blood. This method depends upon acetone extraction and subsequent oxycellulose concentration of the hormone. Recoveries of 80-85 p.c. are reported.

Attempts to isolate this hormone from the serum or plasma of blood to which the hormone has been added have yielded uniformly low recoveries.

The significance of these findings is discussed.

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A METABOLIC ASPECT OF RENAL HYPERTENSION

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Investigations into the aetiology of experimental renal hypertension have been somewhat discouraging despite the fact that an approach to the nature of the condition is readily available in animals by the application of a partial clamp to a renal artery (Goldblatt *et al.*, 1934). The early work of Bright (1836), Tigerstedt and Bergman (1898), Volhardt and Fahr (1914) and others had yielded a substantial body of knowledge associating the kidneys with hypertension. Naturally the clamp procedure by producing a persistently raised blood pressure reorientated attention to the kidney and was adopted in principle for experimentation on a controlled basis. In the search for a causative mechanism, the renal extract of Tigerstedt and Bergman (renin) became suspect. Almost simultaneously the South American (Braun-Menendez *et al.*, 1939) and the North American (Kohlstaedt *et al.*, 1940) schools came to the conclusion that renin was probably an enzyme which acted on hypertensinogen (an α-globulin of plasma) producing hypertensin. The terminology of the North American school differed, but the concept was similar. Defects and deficiencies in the renin hypothesis became apparent and the problem was attacked from many different angles including the possible role of endocrine glands and notably adrenal cortical secretions in the pathogenesis of the condition.

This later swing to seeking extrarenal factors has been marked, and both Smirk (1949) and Pickering (1955) have reviewed and analysed many of these extrarenal contributing factors. The concept of a "mosaic pattern" for the disease is, however, an abnegation of the vital and proven involvement of the kidney in the onset and maintenance of experimental renal hypertension. No conclusive evidence having been produced that known pressor substances cause renal hypertension, other mechanisms within the kidney were explored, amongst which amino-acid metabolism was investigated by studies of renal enzyme systems. After reviewing the data available Bing (1946) concluded that "a definite relationship between renal amino-acid metabolism and experimental renal hypertension has not been discovered."

In this laboratory, *in vitro* and *in vivo* experiments were conducted with

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amino-acids and other substances in a broad attempt to cover many unexplored possibilities, and in 1950 provocative results were obtained. This report presents the early results and subsequent developments.

A Consideration of Some Factors Influencing the Experimental Design.

Although these comments may appear to anticipate the presentation of results, it is felt that greater clarity is achieved by indicating some of the foundations on which the experiments were based.

The defects in the renin hypothesis of experimental hypertension have been summarised by Braun-Menendez *et al.* (1946) and other workers in this field. There are three well-established points of dissimilarity between the action of renin and comparable facets of experimental renal hypertension. Renin exhibits species specificity, repeated injections of renin produce tachyphylaxis and an injection of renin followed by a given dose of adrenaline does not show any sensitisation of the arteriolar bed to adrenaline, and the response is of the same order as that of control animals. On the other hand, a partial clamp applied to a renal artery has produced experimental hypertension in all species of mammals tested; the blood pressure after the initial stages is sustained and there in hypersensitivity to adrenaline. It appears reasonable to assume that these biological differences between renin and experimental renal hypertension would not exist with any causal substance reproducing the effects of the partial clamp.

Three sets of observations entered into the pattern and final elaboration of these experiments:

(1) The substrate—kidney homogenate preparation in our experiments which gave the most consistent rises in blood pressure always contained *dl*-methionine or its derivatives. When grouped amino-acids were used in the mixture, the addition of threonine, valine, aspartic acid and betaine hydrochloride often proved successful.

(2) Mellanby (1946) described the fits in dogs with canine hysteria produced by a diet containing agenised flour. From the clinical description these fits resembled in many respects those exhibited by rats with hypertensive cerebral crises. The agene factor was characterised as methionine sulphoximine (Fortman *et al.*, 1951).

(3) Hartcroft and Best (1949) produced renal damage and the subsequent development of renal hypertension by placing weanling rats on a choline deficient diet for four periods of five days separated by intervals of two days during which some choline was administered. The metabolic relationship between choline and methionine appeared to be of significance.

The first two observations had in common the participation of methionine or a methionine derivative in the experiments involved. The evidence that methionine was an essential component in the third instance was presumptive but more than likely.

Methods.

Adult Wistar rats weighing about 200 gm. were used in the experiments. Hypertension was produced by the clip method of Wilson and Byrom (1941) and the indirect blood pressure was taken from the tail by the apparatus described by Byrom (1947). The direct blood pressure in assay rats was recorded on a slowly moving kymograph drum after heparinisation and cannulation of a carotid artery. The cannula was connected to a mercury manometer of narrow bore capillary tubing (2·0 mm.) the distal limb of which had a float to which the marker was attached. Both the direct and indirect blood pressure readings were what is commonly called the mean pressure. The heart weight in mg. equated to body weight per 100 gm. of rat served as a final check of the blood pressure in hypertensive compared with normal animals.

For convenience, assay rats weighing about 250 gm. were used in the final testing for blood pressure responses. These were unilaterally nephrectomised two weeks before an actual experiment and the remaining kidney was removed before carotid cannulation. Alternately both kidneys were removed just before direct blood pressure determinations and there did not appear to be any difference between the two types of preparation. The important point was to avoid damage to the adrenal glands. Ether on a cotton wool pad was a satisfactory anaesthetic and had the advantage that when an animal was allowed to become lightly anaesthetised the slight struggling on pouring a few more drops on to the pad led to the release of adrenaline and a typical response. Thus the integrity of the adrenals was readily confirmed. Some assay animals were pretreated with desoxycorticosterone acetate, adrenal cortical extract and cortisone by injection 48 and 24 hours before testing.

The kidneys from both normal and hypertensive rats were used extensively as enzyme preparations. Kidney tissue slice (Deutsch, 1936), and kidney homogenate prepared by the method described by Potter (1941), constituted the main suspensions used. In the later experiments homogenate was used exclusively. The substrate mixtures were made up in Krebs' Ringer's Phosphate (K.R.P.), 0·2 ml. containing 0·5 mg. of any amino-acid or an appropriate strength of any other substance, e.g. adrenaline. This volume was added to 2 ml. of the homogenate or to 100 mg. of tissue slice suspended in 2 ml. K.R.P. The preparations were incubated at 39° C. under aerobic and partially anaerobic conditions for periods ranging from 1½ to 4 hours and with a pH range from 4·5 to 5·5. The mixture was then heat inactivated at 60° C. for 15 min., to destroy renin (Helmer and Page, 1939), neutralised, centrifuged and 0·5 ml. of the supernatant injected finally into assay rats, which had been prepared for direct blood pressure recording.

Amino-acids were used either individually or in selected combinations with tissue slice or homogenate. Those groups which gave definite pressor responses were then submitted to further screening tests by elimination and recombination. The amino-acids tested were: glycine, serine, threonine, alanine, aspartic and glutamic acids, leucine, valine, proline, lysine, asparagine, tyrosine, tryptophane, dihydroxyphenylalanine cystine, cysteine and methionine. In addition, methionine derivatives, the sulphoxide and sulphoximine, were employed. Other substances included histamine, adrenaline, pitressin, folic acid, ascorbic acid, adenosine triphosphate, pilocarpine, acetyl choline, commercial adrenal cortical extract, peptic and tryptic digest of gelatin, plasma protein fractions and betaine as the hydrochloride. Plasma proteins were fractionated by ammonium sulphate precipitation (Edsall, 1947). With the plasma protein fractions the formation of hypertensin, which is relatively heat stable, was avoided by heat inactivation of renin before incubation.

RESULTS.

Five successive indirect high blood pressure readings and increased heart weight per 100 gm. of rat body weight were considered essential requirements

to satisfy a rigid standard of classification of hypertensive animals. Table 1 illustrates the difference between normal and hypertensive rats selected at random from a large series.

TABLE 1.

Blood Pressure and Heart Weights.

Type of rat	Number of rats	Blood pressure and S.D.	mg. Heart weight per 100 gm. rat and S.D.
Normal	70	108±17	325±16
Hypertensive	70	177±22	563±74

The kidneys of both normal and hypertensive rats were used in preparing homogenate and tissue slice. Under the experimental conditions employed there was no marked difference between the end results obtained with either type of kidney. It was argued that the activity of a normal kidney preparation under partially anaerobic conditions would approximate to that of a hypertensive kidney under aerobic conditions. This proved to be fallacious reasoning. The pressure-raising properties were practically identical in those instances where positive responses were obtained although partially anaerobic conditions appeared to produce more uniform results and were favoured by the author. The absence of the expected difference might be explained partly by the resilience and consequent return to near normal of the clamped kidney during the time of removal, mincing and homogenising.

Heat inactivation at 60° C. for 15 min. was uniformly successful in destroying renin. This was regarded as important because the presence of renin obscured the interpretation of the results of pilot experiments besides interfering with the basic design. It will have been noted that heat inactivation was generally carried out after incubation.

The hydrogen ion concentration at which the substrate-enzyme system operated in producing a pressor substance was important. All results interpreted as being significant were obtained between pH 4·5 and 5·3. The original range was from 4·5 to 7·5, but was quickly narrowed down to the acid side.

Pretreatment of assay rats with desoxycorticosterone, cortisone and commercial adrenal cortical extract did not make any difference in the nature of positive responses. Any variations were such as one would expect in this type of biological experiment. The results of this pretreatment are regarded as being indecisive as the animals had intact adrenal glands and were treated by only two injections 48 and 24 hours before assay.

No results suggestive of a pressor agent responsible for renal hypertension

were obtained in the experiments with other substances beyond amino-acids and their derivatives.

The kymograph tracings of blood pressure responses are representative of typical experiments in each series. Generally, significant results were followed by many repeated experiments under the same conditions to decide if consistency had been achieved. Fig. 1 shows a typical renin response with repeated injections of kidney homogenate; the shape of the curve, the duration of response and the occurrence of tachyphylaxis are characteristic of renin.

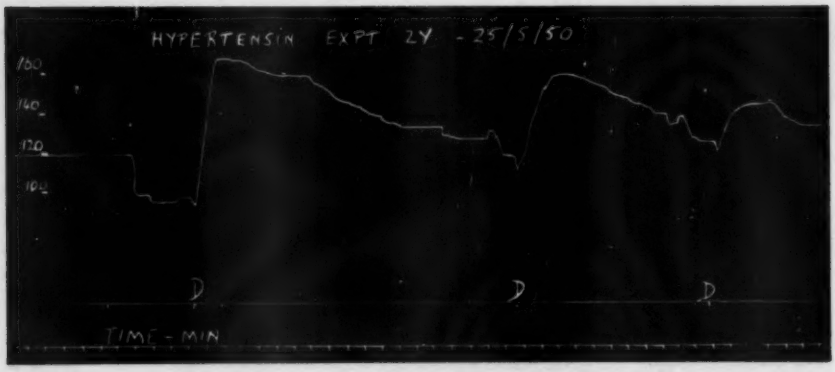

Fig. 1. A characteristic renin response showing tachyphylaxis.

The first intimation of an unusual response occurred when kidney tissue slice was incubated with methionine, betaine hydrochloride, valine, threonine and aspartic acid in K.R.P. ("sol") as substrate. Here the response was sustained and a second injection gave a further rise in blood pressure which was also sustained (Fig. 2). There was no evidence of tachyphylaxis and further experiments confirmed this. Kidney homogenate was then substituted for tissue slice and a more impressive result of a comparable nature was obtained (Fig. 3).

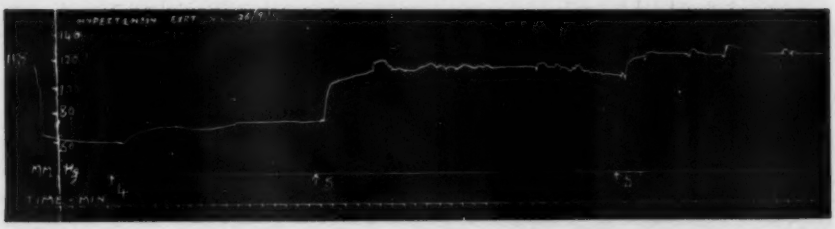

Fig. 2. Responses to "sol" and kidney tissue slice.

Fig. 3. Responses to "sol" incubated with kidney homogenate.

The experiment was repeated many times and the results were provocative, but not conclusive. At this stage, extensive investigation on the possible influence of substrate concentration and of organic and inorganic catalysts were carried out. These variations did not yield any further information than that already on hand. Reverting to the original experiment illustrated in Fig. 3, a complete duplication of the results was obtained. Continuing this line of investigation capricious results, which were difficult to interpret, were obtained, although the general trend of a sustained response with the absence of tachyphylaxis was observed on many occasions (Fig. 4).

Fig. 4. Response of "sol" and kidney homogenate. 1 and 4 are heat inactivated homogenate and 6 is homogenate—"sol" at pH 4·5. Other injections at pH 5·5, 6·5 and 7·5.

The constant common factor of methionine in the substrate mixtures led to the realisation that its derivatives might be implicated in the biological mechanism being investigated. Methionine sulphoxide and methionine sulphoximine in K.R.P. when injected into assay rats gave no pressor responses (Fig. 5). When

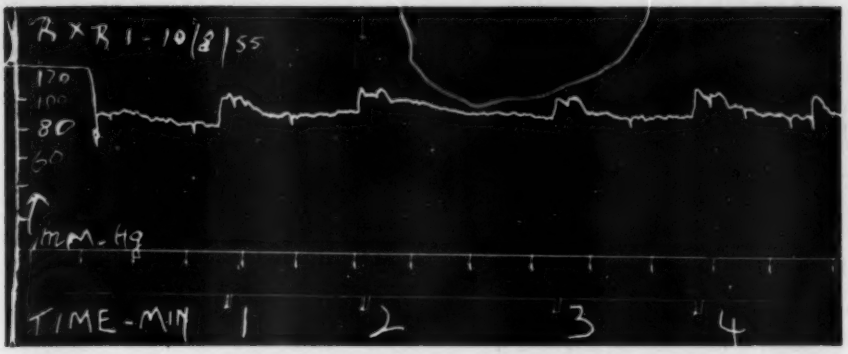

Fig. 5. 1 and 2, methionine sulphoxide in K.R.P.; 3 and 4 methionine sulphoximine in K.R.P.

methionine sulphoxide was incubated with kidney homogenate a characteristic pressor response was obtained, but methionine sulphoximine under the same conditions gave no increase in blood pressure. In addition, when methionine sulphoxide was substituted for methionine in "sol" as substrate, identical responses were obtained (Fig. 6). The results were consistent in twelve experi-

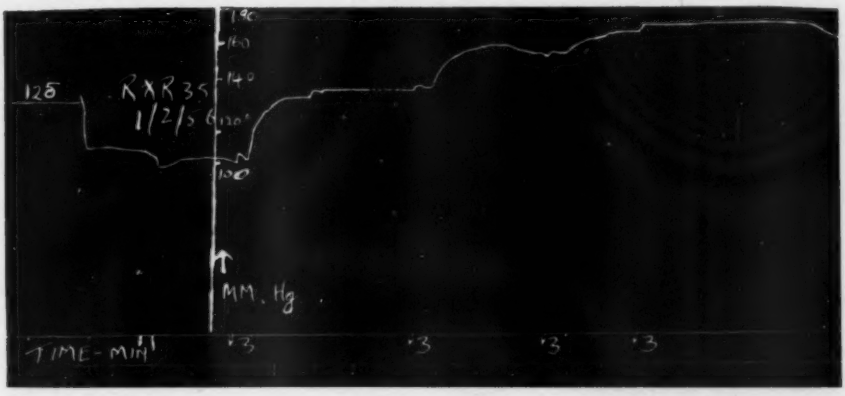

Fig. 6. "Sol" and homogenate but methionine sulphoxide substituted for methionine. 1 is heat inactivated homogenate and 3 is the test substance.

ments in each group; there were also three indeterminate tracings. The rise in blood pressure measured after the second injection ranged from 40 to 70 mm. of mercury. The nature of these responses related back to the earlier results with methionine under the special conditions of the investigation.

An increased sensitivity to the injection of adrenaline was observed in a limited number of experiments after the assay rat had received an injection of successful homogenate-substrate mixture. One μg. of adrenaline injected into such an assay rat produced a response of the same order as that of five μg. injected into a routine assay rat.

DISCUSSION.

The aetiology and pathogenesis of experimental renal hypertension has been open to widespread attack from the time the partial clamp procedure was evolved by Goldblatt *et al.* The bulk of attention was naturally focused on the kidney, but practically every other organ of the body has been subject to close scrutiny, especially the adrenal glands. The majority of scientific workers are adherents of a humoral explanation of a "mosaic pattern," the latter being a term embracing the interactions and balance between all pressor, depressor and neurogenic influences. The mosaic hypothesis does not appeal as an explanation for the cause of hypertension although it embodies the usual mechanisms by which the blood pressure is controlled in non-hypertensive individuals. The humoral postulate is less nihilistic and has a solid foundation in some clinical observations and in the facts emerging from the partial clamping of a renal artery and from such experiments as the removal of a renal artery clip with the subsequent rapid fall of blood pressure to normal (Byrom and Dodson, 1949). The inadequacies of the renin hypothesis have been mentioned and neither hypertensin nor renin possesses the properties one would expect to find in a long acting pressor substance. A similar comment might be made about pepsitensin (Croxatto and Croxatto, 1942). Neurogenic mechanisms, adrenaline, pitressin, tyramine and V.E.M. and V.D.M. (Zweifach and Shorr, 1948) have all been discounted as a continuing cause of raised blood pressure. Exceptions are those cases of pheocromocytoma and isolated instances of experimental neurogenic hypertension (Grimson, 1940) where there have been lability and fluctuations in the levels of blood pressure.

The role of other organs in the pathogenesis of hypertension is difficult to evaluate and an opinion must be based mainly on a consideration of endocrine function. The adrenal medullary tumour, pheocromocytoma, has been causally related to the hypertension that exists in this condition. It appears also that the adrenal cortex plays a part in the hypertension of a rather ill-defined group which includes Cushing's syndrome, adrenal cortical tumour and toxaemia of pregnancy. In two early reports on the treatment of Addisonian patients with desoxycorticosterone, Ferrebee *et al.* (1939) and Soffer *et al.* (1940) noted blood pressures of hypertensive level in several patients treated with large doses of this steroid. Supplementary D.C.A. was found to produce experimental hypertension in rats whose kidneys had been damaged by nephrotoxic serum (Knowlton *et al.*, 1946). In the experiments reported in this paper, pretreatment of assay rats with D.C.A., cortisone and commercial adrenal cortical extract did not influence pressor re-

sponses, but some fallacies are pointed out. There does not appear to be any doubt that the adrenal cortex influences the initiation and course of hypertension, probably in a conditioning capacity or by the exercise of its "permissive" action (Ingle, 1952).

The coincidental facts in the natural history of experimental renal hypertension and human hypertension are most unlikely to be due to chance. Some conditions such as polycystic kidney, external compression of a renal artery (e.g. by tumour or aneurism), unilateral pyelonephritis, coarctation of the aorta, and congenital malformations, may reproduce the effects of a partial clamp on a renal artery. The most common and enigmatic forms of hypertension in man, essential hypertension and the more fulminating malignant hypertension, have a close parallelism to experimental renal hypertension and its sequelae. It might be expected that different forms of treatment would throw some light on the aetiology of essential hypertension. As the majority of drugs used are central nervous system depressants and sympatholytic, adrenolytic or ganglion blocking agents, little information is gained from the point of view of a renal mechanism.

One feature of the experiments reported is the demonstration of a pressor agent from the rat's kidney which possesses properties unlike those of other known pressor substances. The points of difference are the nature of the curve, the sustained response and not only the absence of tachyphylaxis but further rises in blood pressure with repeated injections. The substance when injected produces hypersensitivity to adrenaline. The bio-synthesis of this agent was shown using "sol" as substrate and kidney tissue slice or homogenate as the enzyme system under the experimental conditions detailed. The results were somewhat inconsistent on repetition, but sufficient impetus was given to the study to justify further search for readily reproducible results. In all the amino-acid combinations, methionine was the only amino-acid which was commonly associated with the type of response described. It is interesting to note that methionine is oxidised at pH 5 beyond the disulphide stage *in vitro*, possibly to the sulphoxide (Chinard and Hellerman, 1954). An abnormal toxic metabolite derived from these sources might well contribute to the onset of necrotising arteriolar lesions besides the mechanical effect of the high blood pressure.

The results presented together with collateral evidence suggest that a fundamental derangement of amino-acid metabolism of the kidney is responsible for experimental renal hypertension. The basic mechanism appears to involve mainly methionine and its derivatives.

SUMMARY.

Kidneys of normal and hypertensive rats were incubated in the form of tissue slice or homogenate with a large number of amino-acids and other substances. The resulting mixture was injected intravenously into assay rats to detect any pressor substances produced.

A mixture of methionine, valine, threonine, betaine and aspartic acid as substrate produced impressive but not always consistent results in "step-up" and sustained pressor responses on repeated injection. None of these substances alone or in any other combination gave positive responses when incubated with kidney homogenate or slice.

The substitution of methionine sulphoxide for methionine in the substrate mixture or the use of methionine sulphoxide alone as substrate gave consistent and uniformly reproducible pressor responses.

The deficiencies in the properties of known pressor substances as causal agents in experimental renal hypertension are discussed. It is pointed out that metabolic disturbances of the kidney involving methionine derivatives are likely to figure prominently in the final analysis.

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STUDIES ON THE INTRACELLULAR DISTRIBUTION OF PROTEIN-BOUND AMINO-AZO DYES IN RAT LIVER

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Largely as a result of investigations by Miller and Miller (1947, 1953) into amino-azo dye carcinogenesis, a correlation has been established between the presence and level of dye-binding by certain liver protein(s) and the subsequent development of liver tumours. Nevertheless, even a casual study shows that the relations between these are complex and imperfectly understood. The tumour formation depends on conditions which involve species and organ specificity on the one hand and the effects of various dietary constituents and the nature and position of substituents in the amino-azobenzene nucleus on the other. In all cases a high incidence of tumour formation is associated with high levels of protein-bound amino-azo dyes being reached rapidly in the livers of susceptible animals.

Attention has been directed to the particular proteins which are involved in this phenomenon and various methods have been employed to separate and particularize the participating proteins or the cell components containing them. The technique of differential centrifugation of liver homogenates (to obtain separation of cellular particulates) has been applied to homogenates of livers of rats fed amino-azo dyes (Price, Miller and Miller, 1948; Price *et al.*, 1949a, 1949b and 1950; Hultin, 1956) the resulting fractions being analysed for protein, nucleoprotein, riboflavine and bound amino-azo dye content.

Hultin (1956), in experiments in which a single large dose of dye was given, found that though the early site of dye incorporation was the small granular fraction, subsequently, the soluble cytoplasmic proteins showed the highest levels of bound dye. Price *et al.* (1948, 1949a, 1949b, 1950) observed that, in rats fed the dyes over periods of one week to several months, the highest levels of bound dyes were found consistently in the soluble cytoplasmic protein and small granular fractions, with smaller amounts in the large granular and nuclear fractions.

In view of the possibility, despite the sucrose media used in the above investigations, of microsomal agglutination causing contamination of the other particulate fractions, with consequent apparent increase in the bound dye content of the nuclear and large granular fractions, it was decided to investigate the results of centrifugal fractionation of homogenates using a dispersing agent

such as polyvinylpyrollidone (PVP) and 0·25M sucrose as the suspending medium. Use of PVP and sucrose media for facilitating separation of cell particulates has been reported by Woods (1954) and Novikoff (1956). Judging by RNA content and esterase activity of the mitochondrial fraction, it would appear that Novikoff obtained better separation of mitochondrial and microsomal fractions of rat liver.

Methods.

Sprague Dawley rats, descended from a Holtzman strain, weighing 150-200 gm. were fed the following basal diet:

Casein (ethanol and water washed) (Miller *et al.*, 1944)	200 gm.
Sucrose	710 gm.
Salt mixture (Hubbell, Mendel and Wakeman, 1937)	40 gm.
Maize oil	50 ml.
Halibut oil	2 ml.
α-Tocopherol	0·1 ml.
Aneurin hydrochloride	5·0 mg.
Calcium panthothenate	10 mg.
Nicotinamide	10 mg.
Folic acid	0·5 mg.
2-Methyl-1-4-naphthoquinone	1·0 mg.
Pyridoxine hydrochloride	2·5 mg.
p-Aminobenzoic acid	100 mg.
Inositol	500 mg.
Choline	30 mg.
Vitamin B_{12}	30 γ

Once a week the rats were fed strips of filter paper as a source of vitamin-free cellulose.

The diet was modified to contain 2·0 mg. riboflavine per kg. as determined fluorometrically and either with no amino-azo dye as a control or 0·06 p.c. of either 4-dimethylaminoazobenzene or 3′-methyl-4-dimethylaminoazobenzene. This last was synthesized by the method of Giese, Miller and Baumann (1945), and recrystallized from ethanol, and had a melting point of 116-117° C.

After four weeks on basal diet, or basal diet plus 4-dimethylaminoazobenzene, or two weeks on basal diet plus 3′-methyl-4-dimethylaminoazobenzene, the rats were anaesthetized with ether and the livers perfused *in situ* with 20 ml. of an aqueous solution of 0·15M sodium chloride *via* the portal vein. The liver was then excised and a 10 p.c. homogenate made in freshly prepared ice cold 10 p.c. PVP and 0·25M sucrose, which had been adjusted to pH 7·5-8·0 by the cautious addition of sodium hydroxide. A Potter-Elvehjem glass homogenizer was used in making the homogenate. This was carried out in the cold room at 1-2°. The final pH of the homogenate was 6·9-7·2. Initial supplies of PVP were obtained from May and Baker as 30 p.c. W/V solution containing 0·1 p.c. chlorocresol as preservative. A further supply, of molecular weight 10,000-40,000, was obtained from L. Light & Co.

The homogenate was filtered under suction through glass wool and then centrifuged at 900 *g.* for 7 minutes in an MSE refrigerated centrifuge. The supernatant was decanted and the pellet resuspended in fresh PVP and sucrose medium and recentrifuged as above. This was repeated until examination by phase contrast microscopy showed that a satisfactory nuclear fraction had been obtained; usually 3 or 4 washings were necessary. This provided the nuclear fraction.

The combined washings and supernatant were then centrifuged in a Spinco preparative ultracentrifuge at 15,000 *g.* for 15 minutes. The supernatant was decanted and the pellet, resuspended in fresh medium and recentrifuged twice as above, finally constituted the large granular fraction.

The combined washings and supernatant were then centrifuged at 78,000 *g.* for one hour, the supernatant decanted and the small granular pellet obtained giving the small granular fraction. All relative centrifugal forces are stated for centre of the tube and the times do not include the period of acceleration and deceleration of the centrifuge.

The proteins were precipitated by boiling for 3 minutes after adding 10 ml. of 1M acetate buffer pH 5, and 10 ml. of distilled water to each particulate fraction, and an aliquot of 1M acetate buffer pH 5 to the soluble cytoplasmic protein fraction. The precipitates were collected on hardened filter papers, washed with the above buffer and ethanol, and then extracted with ethanol in a Soxhlet apparatus as described by Miller and Miller (1947).

The proteins, after drying *in vacuo* first over $CaCl_2$ and finally over H_2SO_4, were weighed, and 25-50 mg. quantities subjected to ethanolic KOH hydrolysis at 80° C. for 20 hours. The bound dyes with "polar" properties (as described by Miller and Miller, 1947) were then extracted according to their method and estimated spectrophotometrically at 520 mμ using a Unicam SP550 spectrophotometer. In general, observations were made in duplicate.

RESULTS.

Protein distribution.

The distribution of proteins is shown in Table 1. The livers of rats fed amino-azo dyes showed a decrease in total protein. The nuclear fraction showed an increased protein content and the large granular fraction a decreased con-

TABLE 1.

Distribution of protein in rat liver fractions (mg. protein/gm. fresh liver not corrected for loss on glass wool).

Liver fraction	Basal diet (4 weeks)	Basal diet + 4-dimethylamino-azobenzene (4 weeks)		Basal diet + 3′-methyl-4-dimethyl-aminoazobenzene (2 weeks)
	(1)	(2)	(3)	(4)
Whole homogenate	112	98	100	96
Nuclear fraction	14	20	21	21
Large granular fraction	28	19	20	18
Small granular fraction	18	15	15	15
Supernatant fluid	48	40	38	38

tent. Because of loss of protein through adsorption and retention on the glass wool, no absolute values could be given to the protein content of each fraction. To determine the protein loss occasioned by filtering the homogenates through glass wool, aliquots of an homogenate of normal rat liver were (a) unfiltered and (b) filtered through similarly packed glass wool. The proteins were then precipitated and extracted as before and the dried proteins weighed. It was

found that retention by the glass wool accounted for a loss of 10 mg. of protein per gm. of fresh liver. This loss was distributed thus:

nuclear fraction	$3{\cdot}5 \pm 0{\cdot}5$ mg.
large granular fraction	$2{\cdot}5 \pm 0{\cdot}5$ mg.
small granular fraction	$1{\cdot}5 \pm 0{\cdot}5$ mg.
soluble protein fraction	$2{\cdot}5 \pm 0{\cdot}5$ mg.

Bound amino-azo distribution.

The amino-azo dye levels were measured spectrophotometrically in acid-ethanol at 520 mμ, and expressed as log. Io/I per 100 mg. protein. To determine if there was any selective retention by the glass wool, of dye-bound or dye-free protein in the soluble cytoplasmic fraction, aliquots of an homogenate of dye-fed rat liver were taken through the entire procedure with or without filtering through glass wool. It was found that this caused no alteration in the extinction at 520 mμ per 100 mg. soluble cytoplasmic protein.

Liver protein samples of rats fed the basal diet and taken through the same procedure showed non-specific absorption in the liver extracts.

The following corrections for non-specific absorption at E_{520}. per 100 mg. protein were applied and corrected values for specific absorption due to protein-bound dye are shown in Table 2.

Whole homogenate	0·04
Nuclear fraction	0·06
Large granular fraction	0·05
Small granular fraction	0·04
Supernatant fluid	0·03

TABLE 2.

Bound-dye levels in rat liver expressed as E_{520} per 100 mg. protein (corrected for non-specific absorption).

Liver fraction	Diet containing 4-dimethylamino-azobenzene		Diet containing 3′-methyl-4-dimethylaminoazobenzene
Whole homogenate	0·38	0·35	0·30
Nuclear	0·19	0·21	0·20
Large granular	0·12	0·13	0·15
Small granular	0·41	0·44	0·34
Soluble proteins	0·52	0·51	0·37

DISCUSSION.

In accordance with other reports of investigations of intracellular distribution of bound amino-azo dyes, no cellular fraction was obtained here which was free of bound dye. However, the levels of bound dyes found in the large granular fraction were consistently lower than those found by Price *et al.* (1948, 1949a,

1949b, 1950). This could be attributed to a decrease in small granular contamination of the large granular fraction. PVP was included in the suspending medium in order to reduce such contamination because of its effect in decreasing particle agglutination, and hence increasing differential sedimentation. It is not practicable and probably misleading to make a direct comparison between the results of Price *et al.* and those reported here because of differences in suspending media and centrifugal forces employed. This is also reflected in the differences of protein content of the various fractions as compared with the protein content of the fractions obtained by Price *et al.*

SUMMARY.

Fractionation of liver cell particulates prepared from rats fed amino-azo dyes was carried out by differential centrifugation using PVP and sucrose media.

Protein-bound dye levels were estimated in each fraction; and although, as other observers have found, bound dye was detected in all fractions, the level of bound dye in the large granular fraction was relatively lower than that previously recorded. This could possibly be explained as due to less contamination of the mitochondrial fraction by microsomes. An attempt was made to decrease such contamination by incorporating PVP in the suspending medium to reduce microsomal agglutination.

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THE INVASION OF THE CHICK EMBRYO BY ECTROMELIA VIRUS AFTER INOCULATION ON THE CHORIOALLANTOIC MEMBRANE

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Both Paschen, and Burnet and Lush, in 1936 independently reported that ectromelia virus grew on the chorioallantois of the developing egg and usually brought about the death of the embryo if incubation was prolonged for four or five days. Burnet and Lush also detected virus in the liver of the embryonic chick as early as 24 hours after inoculation and noted scattered areas of necrosis which developed later in both liver and spleen. The rapidity with which virus reaches the embryo makes it probable that the virus particles are carried directly *via* the blood stream but the possibility remains that they might make their way to the embryo by the more circuitous route of infection from cell to cell and tissue to tissue; for instance, virus might be liberated from the allantoic side of the chorioallantois into the allantoic fluid and then infect the amniotic sac and finally the embryo. The possibility of some such indirect route has been emphasised by Köhler's demonstration (1955) of the existence, in some cases at least, of a direct communication between the allantoic and amniotic cavities. Himmelweit (1938), by an ingenious technique, was able to observe directly the viruses of ectromelia and vaccinia while they were growing in the cells of the chorioallantois. He found that the lesions were associated with the larger blood vessels and considered that the site of primary attack for the virus might be some type of cell belonging to the vessel wall. Virus particles would then be liberated directly into the blood stream and the embryo could be infected by this route. However, he also described two other phenomena that show that a more indirect route is possible; one, the cells at the edge of a lesion tended to be in the earliest stage of infection which suggests that infection spreads from cell to cell—at least within a lesion—and two, globular bodies containing virus were liberated from the cytoplasm of infected cells into the intercellular spaces. More recently, Noyes and Watson (1955) were able to follow the increase of vaccinia virus in cultured human cells by means of the fluorescent antibody technique and noted the presence of antigenic material in cytoplasmic processes. They considered that these processes might release free virus extracellularly or else transmit it directly to other cells.

It appears certain that cell to cell transmission does occur in virus infections of a variety of tissues, although Dulbecco (quoted by Ackermann and Francis, 1954) in his studies of plaque formation in cellular monolayers *in vitro* produced some evidence in favour of the simple diffusion of virus particles in the overlay. Pock formation on the chorioallantois is an obvious example of direct cell to cell transmission, although it must be admitted that even in this case a film of moisture or tissue fluid might play the part of an overlay.

Another example seems to be provided by Weller's demonstration (1953) that agents isolated from cases of varicella and herpes zoster will produce cytopathogenic changes in roller tube cultures affecting as many as 90 p.c. of the cells in spite of the fact that the fluid medium remains non-infective; serial passage is only successful if tissue suspensions are used.

This work is concerned with the spread of ectromelia virus through the developing egg as revealed by a series of titrations on different tissues at various intervals after inoculation onto the chorioallantoic membrane.

Materials and Methods.

Virus.

The Moscow strain (Fenner, 1947). A stock suspension of virus in 50 p.c. V/V glycerol saline was made from heavily infected chorioallantoic membranes inoculated with a mouse liver suspension of virus and was stored at $-20°$ C.

Method of titration.

(a) In eggs. The procedures described by Beveridge and Burnet (1946) for inoculating the dropped chorioallantoic membrane of 11 or 12 day eggs were followed. Tenfold dilutions of the virus suspension in 10 p.c. horse serum saline were inoculated onto batches of 5 or 6 eggs, which were incubated for 3 days at 35° C. when the inoculated chorioallantoic membranes were harvested and the pocks counted with the aid of a dissecting microscope (total magnification 5 or 10 ×). The results are expressed as infective units (i.u.) per gm. wet weight of tissue or per ml. of allantoic fluid, amniotic fluid or blood.

(b) In mice. Groups of 3 or 4 mice were inoculated intraperitoneally with a series of tenfold dilutions of virus suspension. All survivors were killed after 13 or 14 days and their sera examined for antihaemagglutinins following the technique laid down by Fenner (1947). The ID_{50} was then calculated by the method of Reed and Muench and the results converted into the equivalent titre on eggs by dividing by 10 (cf. Fenner, 1948, 1949).

Harvesting of eggs.

Blood was collected from vessels at the air space end of the egg by the technique detailed by Beveridge and Burnet (1946), and usually the opportunity was taken to collect some allantoic fluid at this stage. Next, each egg was separated into two halves by cutting through shell, shell-membrane and chorioallantois with a pair of scissors around the equator, and the bottom half together with the embryo was allowed to fall away from the inoculated side. The amniotic fluid was collected first, followed by the embryo, the amniotic membrane, the yolk sac and the uninoculated part of the chorioallantois. The embryo and its membranes were well washed in saline and the brain and liver removed aseptically from the embryo. The various membranes and organs were pooled, weighed, and ground with physiological saline in a mortar which had been precooled to $-20°$ C.; the tissues immediately

froze and the ice crystals that formed helped in their homogenisation. The homogenate was spun in an angle centrifuge at 700 *g* for 10 minutes and the supernatant kept at − 20° C. until the titrations could be performed.

EXPERIMENTAL.

Anderson (1954) showed that the titre of either vaccinia or influenza virus was diminished if ground with alundum or powdered glass or even if ground in a mortar without added abrasive. This loss of viability was demonstrated both when the virus suspension was ground by itself and when ground in the presence of normal chorioallantoic membrane. However, he found that virus could be recovered without loss of titre by the use of a metal mincer which disrupted the tissue and its cells by means of shearing stresses. Strangely enough, this technique recovered no more virus from infected tissue than did the usual method of grinding in a mortar. The infected tissue comprised vaccinia-inoculated chorioallantoic membrane harvested after 48 hours' incubation and chick embryo lungs harvested 3 days after inoculation with influenza virus.

Since, in the present investigation, tissues were to be titrated to determine their virus content quite early in the growth cycle, it was thought advisable to check whether the prechilled mortar method of homogenisation exerted a deleterious effect on the viability of ectromelia virus. A virus suspension obtained by homogenising infected chorioallantoic membrane was diluted in serum-saline and ground by the prechilled mortar method both alone and after the addition of chorioallantoic membrane. After centrifuging at 700 *g* for 10 minutes the homogenate was titrated on eggs. These results, when compared with the expected virus titre calculated from the concentration of virus in the original suspension, did not indicate any loss of virus. The prechilled mortar method was then compared with several other homogenisation techniques by grinding aliquots of a virus suspension with normal chorioallantoic membrane and titrating the resulting homogenate after centrifugation. The results which are set out in Table 1 were rather variable but the prechilled mortar method gave a reason-

TABLE 1.

Recovery of ectromelia virus added to normal chorioallantoic membrane after various methods of homogenisation.

Method of homogenisation	Titre of viable virus (i.u. per ml. of suspension)	
	Series 1	Series 2
Prechilled mortar	$14{\cdot}0\times10^{3}$	$13{\cdot}8\times10^{4}$
Porcelain mortar and alundum	$5{\cdot}6\times10^{3}$	$4{\cdot}2\times10^{4}$
Teflon tissue homogeniser	$13{\cdot}2\times10^{3}$	$29{\cdot}8\times10^{4}$
Ten Broeck tissue homogeniser	$18{\cdot}8\times10^{3}$	$16{\cdot}0\times10^{4}$
Control	$15{\cdot}2\times10^{3}$	$23{\cdot}0\times10^{4}$

ably good recovery of viable virus; the result in series 2, Table 1 ($13{\cdot}8 \times 10^4$ i.u. per ml. compared with $23{\cdot}0 \times 10^4$ i.u. per ml. for the control) was the lowest recovery found in any experiment. Grinding the virus with alundum caused the greatest loss of virus of any of the homogenisation procedures tried, but the loss was not as great as that found by Anderson in the case of vaccinia virus. A comparison of the prechilled mortar technique with a Waring-type blendor by homogenising material similar to that used before and also infected membrane harvested after 16 hours' incubation yielded results slightly in favour of the former ($14{\cdot}6 \times 10^2$ as against $8{\cdot}9 \times 10^2$ i.u. per ml. and $2{\cdot}0 \times 10^3$ as against $1{\cdot}1 \times 10^3$ i.u. per membrane). No more virus could be liberated from homogenised infected membranes by shaking at 4° C. or 37° C., by trypsin digestion or by the use of an ultrasonic oscillator (9 kilocycles per sec.). It was therefore decided that the use of the prechilled mortar technique followed by a clarifying spin at 700 *g* for 10 minutes would give a satisfactory recovery of virus from infected tissues.

As a preliminary experiment six eggs were inoculated on the chorioallantoic membrane with 3×10^4 i.u. of ectromelia virus and incubated at 35° C. After 3, 4 and 5 days' incubation, pairs of eggs were harvested, the various tissues homogenised and infectivity titrations performed. The results are set out in Table 2. The eggs harvested after five days were dead and gave somewhat lower titres than those harvested after four days. As it is possible that they had been dead for some time and as the results add nothing of any significance, they have been omitted from the Table. However, it may be mentioned that the chorioallantoic membrane from the bottom half of these eggs (i.e. the uninoculated portion of the chorioallantois) was titrated and gave a value of $9{\cdot}4 \times 10^5$ i.u. per gram of tissue, although no pocks were visible upon it.

TABLE 2.

The distribution of ectromelia virus in the infected chick embryo after inoculation on the chorioallantois. The values are expressed in i.u. per gm. or per ml.

Period of incubation (days)	Portions of the egg					
	Allantoic Fluid	Amniotic Fluid	Amniotic Membrane	Yolk Sac	Liver	Brain
3	$3{\cdot}6 \times 10^3$	not done	$2{\cdot}2 \times 10^5$	$6{\cdot}1 \times 10^5$	$2{\cdot}2 \times 10^5$	$3{\cdot}3 \times 10^3$
4	$1{\cdot}1 \times 10^3$	$3{\cdot}4 \times 10^3$	$9{\cdot}0 \times 10^5$	$1{\cdot}4 \times 10^5$	$2{\cdot}8 \times 10^4$	$2{\cdot}3 \times 10^5$

From the Table it is clear that virus invaded all the tissues examined and probably multiplied there, but its titre in both amniotic and allantoic fluid remained low, particularly in the latter.

In order to follow the progress of the virus from the chorioallantoic membrane to the various organs or membranes of the embryo in more detail, a batch

of eggs, inoculated with $3{\cdot}6 \times 10^4$ i.u. per egg, was harvested in lots of three after incubation for 8, 12, 16, 20, 24, 32, 48, and 72 hours. Blood, allantoic and amniotic fluids, chorioallantoic and amniotic membranes, liver and brain were collected, pooled, homogenised with saline where necessary, and titrated in eggs to give the results set out in Table 3. If no pocks were detected by chorioallantoic inoculation the material was titrated in mice. All results in the Table are given in terms of egg infective units by dividing the results of the mouse titrations, expressed in ID_{50}, by ten.

TABLE 3.

The distribution of ectromelia virus in the chick embryo at various times after inoculation on the chorioallantois. The values are expressed in egg i.u. per gm. or per ml.

Portion of egg	Period of incubation							
	8 hr.	12 hr.	16 hr.	20 hr.	24 hr.	32 hr.	48 hr.	72 hr.
Blood	<2†	>4	$6{\cdot}3 \times 10$	8	$1{\cdot}1 \times 10^2$	$5{\cdot}2 \times 10^3$	$9{\cdot}9 \times 10^3$	serum $6{\cdot}2 \times 10^3$ cells $2{\cdot}9 \times 10^4$
Allantoic membrane (minus the site of inoculation)	neg.*	neg.	<2	4	6	$6{\cdot}6 \times 10^2$	$1{\cdot}5 \times 10^5$	$1{\cdot}3 \times 10^6$
Amniotic sac	neg.	neg.	neg.	neg.	<2	$1{\cdot}1 \times 10^2$	$9{\cdot}6 \times 10^4$	$3{\cdot}3 \times 10^6$
Brain	neg.	neg.	neg.	neg.	neg.	$1{\cdot}1 \times 10^2$	$4{\cdot}8 \times 10^4$	$2{\cdot}8 \times 10^6$
Liver	neg.	<8	$1{\cdot}4 \times 10$	$1{\cdot}4 \times 10^2$	$1{\cdot}4 \times 10^3$	$3{\cdot}2 \times 10^4$	$3{\cdot}4 \times 10^7$	$4{\cdot}5 \times 10^7$
Allantoic Fluid	neg.	neg.	neg.	neg.	2	9	6	9

† A figure indicates that virus was detected.
* Negative indicates that 0·05 ml. or gm. inoculated into each of 4 mice failed to infect them.

The most striking feature of the results is the fact that virus was found in the blood at the earliest time that it was examined, namely, after 8 hours' incubation, and in the liver after only 12 hours' incubation.

DISCUSSION.

It seems clear that ectromelia virus finds its way rapidly from the chorioallantois into the blood stream and is then presumably carried to all tissues. This virus, when multiplying on the chorioallantois, first shows a definite increase in titre after 10 to 12 hours (unpublished results) so that the virus detected in the blood at 8 hours may have represented some of the original inoculum which had not passed into the eclipse phase. The liver of the embryo contained virus after 12 hours while the chorioallantoic membrane (apart from the site of inoculation) and the amniotic sac showed little evidence of virus multiplication in the first 24 hours and the titre of virus in the allantoic fluid was also very low. Thus it seems evident that the embryo was infected *via* the blood stream. Presumably the virus multiplied in all tissues and organs examined

since the final titres were fairly high. For comparison, the titre of the inoculated portion of the chorioallantoic membrane infected by a comparable inoculum is about $1\text{-}4 \times 10^8$ per gm. after 3 days' incubation.

Some attempts to adapt ectromelia virus to growth in the cells lining the allantoic cavity by suballantoic inoculation and passage of the allantoic fluid were unsuccessful. The injected virus multiplied in the allantoic and amniotic membranes but very little virus was released into the allantoic fluid which probably explains the failure to passage the virus by this means. Also, for this reason, it is unlikely that virus in allantoic fluid or amniotic sac plays any part in the transfer of infection from chorioallantois to embryo.

There is good evidence that certain other viruses do spread from the site of inoculation on the chorioallantoic membrane to the embryo *via* the blood stream. For instance, Burnet and Fraser (1952) showed that this was the case with Influenza A virus, both with a strain adapted to growth on the chorioallantois (NWS) and with a more typical strain (MEL), which is well adapted to allantoic passage. Fox and Laemmert (1947) considered that their results with the virus of yellow fever indicated an early multiplication at the site of inoculation with rapid invasion of the blood and infection of the embryo.

The evidence obtained from studies made on the related virus of vaccinia is not inconsistent with the view that the virus is transferred by the blood. The observations of Himmelweit (1938) and Noyes and Watson (1955), as mentioned in the introduction, could support either the hypothesis of direct transmission *via* the blood or indirect transmission from cell to cell or to tissue space. Tobin (1956) in a study of the distribution of vaccinia virus in the developing egg after inoculation on the chorioallantoic membrane, determined the virus concentration in various tissues but did not include blood. She considers that the virus may have grown in the cells lining the allantoic cavity since it increased in amount in the allantoic fluid. On the other hand, although virus was present in the amniotic fluid even after one day, it did not subsequently increase in amount. Her results, where they can be compared, are fairly similar to those reported here with the exception that she found quite a high titre of virus (approximately 10^5 i.u. per ml.) in the allantoic fluid after 3 days. Buddingh (1936) studied sections of a series of infected embryos harvested from 1 to 7 days after inoculation and thought that the virus passed *via* the blood to the embryo since the lesions appeared to originate as perivascular infiltrations about the smaller blood vessels. He remarked that the inoculum was so great (it consisted of small pieces of heavily infected chorioallantois which were then placed direct on the exposed chorioallantoic membrane of 14-day eggs) that the virus possibly passed direct from inoculum to blood stream, although he considered it more likely that it was disseminated only after production of a local lesion. The results reported here show that the virus probably can pass directly from the inoculum into the blood without previous multiplication when the inoculum consists of a suspension of virus.

SUMMARY.

Infectivity titrations on various tissues and organs of the developing chick embryo at intervals after inoculation on the chorioallantoic membrane have shown that the virus appears to pass directly *via* the blood stream to the embryo and to multiply in those organs and extra-embryonic membranes examined, namely: liver, brain, chorioallantois, amniotic sac and yolk sac.

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EXPERIMENTAL VECTORS OF THE FIRST LARVAL STAGE OF *DIBOTHRIOCEPHALUS LATUS* (CESTODA) IN AUSTRALIA

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Dibothriocephalus latus (Linné) 1758[1] is a rare human parasite in Australia and it is not established as an endemic infection anywhere on this continent. Occasional cases are found in immigrants from the endemic areas of Europe and one of them provided an opportunity to test some of the autochthonous copepods as vectors of the first larval (procercoid) stage.

Materials and Methods.

After treatment with atebrin between 5 and 6 metres of segments of *D. latus*, but not the head, were passed by a North Italian immigrant of nine months residence in Australia. The patient thought he had acquired his worm about a year before coming to Australia.

Stained segments and serial sections agreed in morphology with the criteria accepted by Mueller (1937) and by Faust (1952) for this genus and species. The uterus was coiled in "rosette" pattern, the vagina and cirrus terminated in a common genital pore and this and the uterine pore had separate openings on the ventral surface. The cirrus and seminal vesicle were divided by a constriction as shown by Mueller (1937). Eggs were regularly oval; the means of 20 measurements of length and breadth were 71 by 53 μ with ranges of 81 to 67 and 56 to 49 μ. The coracidia when hatched moved with the leisurely rolling movement characteristic of *Dibothriocephalus*.

Eggs from the faeces were washed in dilute copper sulphate solution and in water and stored in a cool dark cupboard at room temperature. Portions of this sediment were withdrawn at intervals and incubated at 25° C. for the development of coracidia.

Copepods were collected from small lakes of a few acres in the suburbs of Sydney; they were kept in small glass jars or excavated blocks at room temperature. The copepods were examined for infections by stranding them on glass slides, or if a better view was needed, by mounting them under a cover-slip supported by short lengths of horsehair.

The Cyclopoida were identified from the descriptions given by Sars (1914) Gurney (1933) and Henry (1922); in the last publication *B. minuta* and *G. brevicornis* are also described. A key for the genus *Boeckella* is given by Fairbridge (1945) and for males of the genus *Gladioferens* by Nicholls (1944). The drawings of *G. brevicornis* by Henry are incomplete, but this deficiency has been remedied by Dakin and Colefax (1940).

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[1] *Dibothriocephalus latus* (Lühe) 1899 and *Diphyllobothrium latum* (Lühe) 1910 are synonymous. Wardle and McLeod (1952) discuss these two generic names and conclude that the former is correct.

RESULTS.

The infection rates in the copepods tested are given in Table 1. The "positive" column indicates the numbers of individuals which had procercoids in the body cavity and takes no account of the stage of development of the procercoids. The longest period of observation was 24 days for *Boeckella minuta*.

TABLE 1.

Results of experimental infections of copepods with coracidia of Dibothriocephalus latus.

Species of Copepod	Positive	Negative	Remarks
ORDER *CALANOIDA*			
Boeckella minuta	10	10	all living and well developed; several multiple infections;
Gladioferens brevicornis	8	32	some multiple infections; development retarded as compared with Boeckella.
ORDER *CYCLOPOIDA*			
Mesocyclops leuckarti	2	94	only early stages, in body cavity.
Leptocyclops agilis	1	35	procercoids dead in body cavity and in gut wall.
Cyclops varicans	1	10	procercoid dead in body cavity.
Platycyclops sp	1	16	early stage, in body cavity.
Pachycyclops annulicornis	0	11	
Halicyclops aequoreus	1	3	4 procercoids, alive, early stages, in body cavity.
Species unknown	1	172	early stage procercoid, body cavity.

The heading "species unknown" includes immature specimens from mixed cultures, mostly of *M. leuckarti* and *L. agilis*. In the Cyclopoida, the infections were of undeveloped procercoids which moved sluggishly in the body cavity. Many of them were dead in the body cavity and one was seen dead in the outer coat of the stomach wall.

The development of the procercoids in the single infected *Halicyclops* was not followed through as it could not be found again in later inspections of the culture.

In *Boeckella* the procercoids seemed to develop normally up to the formation of the head grooves, the calcareous granules in the body and the cercomer. Unsuccessful efforts were made to infect fish of three species, *Gambusia affinis*, "hardy-heads" (*Atherinosoma microstoma*) and young goldfish (*Carassius auratus*) with these infected copepods.

DISCUSSION.

The possibility of *D. latus* becoming endemic in Australia has been discussed by Sandars (1951) and by Backhouse and Bearup (1951). The present rapid increase of population and resources in this country have made changes in two

directions which are of interest to the present enquiry; one is the greater number of immigrants from European countries where *D. latus* is endemic, the other is the increase of the area of freshwater lakes by the building of large new storage dams.

The records of infections with this worm in Australia were published by Sandars (1951), and three additional cases have come to our notice since then; all were migrants, one each from Esthonia, Ireland and North Italy. In 1939 Gordon reported *D. latum* infections in three dogs in New South Wales; the worms were identified for him by Dr. H. A. Baylis of the British Museum of Natural History. Mr. Gordon kindly gave me a worm from one of these dogs, some proglottides were stained and others were cut in serial sections; after careful examination of this material I feel satisfied that, at the present time, this worm would be included in the genus *Spirometra.* My reasons for thinking so are that the vaginal opening is distinct from and posterior to that of the cirrus pore, the uterus is spirally coiled and within it the eggs are of the typical Spirometrid shape with pointed ends. My own experience leads me to agree with Mueller (1937) that these characters are constant for *Spirometra* and are sufficient to separate the "Spirometra complex" (which includes the species *Dibothriocephalus* (now *Spirometra*) *erinacei, mansoni, raillieti,* etc.) from the "Dibothriocephalus complex" (*D. latus,* etc.) of species. Worms of the *Spirometra* group from cats, dogs and foxes in Australia have usually been named specifically as *S. erinacei* and their plerocercoid stages are common in frogs, snakes and lizards, and in wild pigs. No locally acquired case of *D. latus* infection in man has ever been reported.

Lakes seem to be essential for the life cycle of *D. latus,* for only there are found together suitable genera of copepods and fish to act as first and second intermediate hosts. The extensive lake systems of northern Asia and Europe are endemic centres of long standing. The Great Lakes of North America may have been free of infection until the closing years of last century when many migrants from Baltic countries came to work in the iron mines of Minnesota and Michigan; from 1906 onwards locally acquired infections came to notice in the surrounding districts.

In the last few years the Southern Hemisphere has acquired an endemic centre in Lake Colico, Chile. Faust (1952) says that many likely carriers from north Europe have recently built holiday cottages on the shores and the sewage from them is discharged into the Lake. Trout, also recently introduced from Europe, act as second intermediate hosts. Elsewhere the larger carnivorous fish, such as perch and pike seem to be more suitable vectors and in some North American Lakes, which receive raw sewage from lakeside settlements, nearly every one of these fish is infected.

The hosts for the first (procercoid) stage of *D. latus* in Europe and North America are copepods of the genus *Diaptomus.* This genus is widespread and

abundant in open waters of pools and lakes in the temperate zone of the Northern Hemisphere; it gets less common in hot climates but some species extend across the equator and two are found in tropical Australia (*D. orientalis* Sars and *D. lumholtzi* Brady). In the South Temperate zone the genus *Diaptomus* is replaced by related Calanoid genera, including *Boeckella* and *Gladioferens*. Both of these genera (and *Diaptomus*) prefer open water clear of the weedy fringes; this brings them within the feeding range of small fish which might act as carriers of the second larval (plerocercoid) stage. Transmission to man depends on the ingestion of fish containing plerocercoids which have not been killed by heat in the cooking process.

Several species of Cyclopoida are named in text-books as vectors of *D. latus*, but the original papers show that although they ingest the coracidia, and sometimes do become infected, they are not nearly as efficient as *Diaptomus* and are probably abnormal hosts. All species of Cyclopoida which were examined by me had fed on coracidia; in most of them the coracidia were already dead, or had lost their epithelial coat, but were still moving sluggishly in the foregut. In many uninfected copepods the larval hooks in the gut or faeces were the only signs that opportunities for infection had occurred. A few oncospheres got so far as to penetrate the gut wall but died there or in the body cavity; they were filled with small granules and were commencing to degenerate, but again the hooks left no doubt of their identity.

Procercoids seemed to develop normally in the body cavity of *Boeckella minuta* and in the later stages had several calcareous granules in the body, a well-developed cercomer and rudimentary head grooves. *Gladioferens* seemed to be less suitable as there was a heavy mortality in partly developed procercoids. *B. minuta* and *G. brevicornis* are widely distributed in New South Wales and the former has also been collected in Victoria (Henry, 1922; Dakin and Colefax, 1940). Other members of the two genera are found throughout temperate Australia (Nicholls, 1944; Brehm, 1953; Fairbridge, 1945).

In the Northern Hemisphere where *D. latus* is endemic as a human parasite, the freshwater lakes are large enough to support a fishing industry. The food fish are carnivorous species and probably acquire their infections by eating smaller fry, which are themselves infected by the copepods. Discharge of sewage into the lakes provides the eggs and coracidia to infect the copepods and as each worm carrier is estimated to provide at least a million eggs each day the heavy infections in fish are not surprising.

In Australia we have few large freshwater lakes and none of them is large enough to support a fishing industry. Artificial lakes now under construction might be large enough to attract a small fishing community as well as the usual sporting anglers, and as *Boeckella* is already present in the storage area, there is a possibility the life cycle of *D. latus* could be completed in them as it has in Lake Colico. The most effective method of preventing this is to prevent the

discharge of sewage into lakes and feeder streams. Regulations to prevent this type of pollution of fresh water are already in existence in New South Wales and probably in all the other States, and the danger could only arise from undetected breaches of the regulations.

SUMMARY.

Boeckella minuta is shown to be a suitable host for the procercoid stage of *D. latus*. *Gladioferens brevicornis* can also be infected, but no mature procercoids were found in the material available. Both these genera belong to the same order (Calanoida) as *Diaptomus*, the vector of *D. latus* in the Northern Hemisphere.

Species of the order Cyclopoida were rarely infected with procercoids of *D. latus* and those procercoids which reached the body cavity seemed to be in unfavourable conditions and many were dead.

Reasons are given for believing that Dibothriocephalid worms from dogs in New South Wales, which were previously reported to be *D. latus*, belong to the genus *Spirometra*.

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